The Tohoku Journal of Experimental Medicine, Vol. 68, No. 2, 1958

The Erythrocytes Sensitized with Tuberculin-Polysaccharide in the Circulating Blood of Children :—Their Diagnostic Application for Tuberculosis in Infancy and Childhood*

(Studies on Tuberculosis in Infancy and Childhood. 3rd Report)

By

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(Received for publication, March 15, 1958)

In the preceding paper[1] we reported that the erythrocytes sensitized with tuberculin-polysaccharide were found in the circulating blood of the rabbits into which the living tubercle bacilli had been introduced intratracheally, and that these sensitized erythrocytes would not be detected after the development of the antibody against the tuberculin-polysaccharide in the serum. It was also reported by us[2] that the adsorption of the tuberculin-polysaccharide to erythrocytes in the blood—the sensitization of erythrocytes with tuberculin-polysaccharide in the blood—would take place in the following conditions: (1) the amount of the tuberculin-polysaccharide in the blood was too much as compared with that of the antibody against the tuberculin-polysaccharide in the serum, or (2), even when the amount of the tuberculin-polysaccharide was not so much, the antibody against the tuberculin-polysaccharide was either poorly formed or was absent in the serum.

In 1956 Török[3] reported that the Middlebrook-Dubos reaction was negative in 78.9% of the infantile cases with active tuberculosis, and he took this finding as an evidence for the poor formation of the antibodies against tubercle bacilli in infants.

It has also been well known that a tuberculous—apparently healthy—adult case is not infrequently detected in the family of the infants with tuberculous infection. So a frequent contact with such a tuberculous patient is common in a usual Japanese family, and it is very often that

* Read at the 12th Meeting of the Aomori Pediatric Society, March 24, 1957, Hirosaki.

a very early stage of tuberculous infection is encountered in the pediatric practice.

As mentioned above, owing to the poor formation of the antibody against tubercle bacilli as well as to the frequent contact with the tuberculous case in the family circle, it may be reasonable to deduce that, in the infantile cases with tuberculous infection, their erythrocytes in the circulating blood will be and keep sensitized with the tuberculin-polysaccharide.

In the present paper, we shall demonstrate the existence of the erythrocytes sensitized with tuberculin-polysaccharide in the circulating blood of the children with tuberculous infection and discuss the relation among the sensitized erythrocytes, Middlebrook-Dubos' reaction and the antibody against the tuberculin-polysaccharide in their serum.

Experimental

Material and Method

1) The materials subjected to this investigation were 278 cases under the age of 13 years who had banal complaints such as anorexia, vomiting, diarrhea and so on. They might or might not be tuberculously infected. On all these children, roentogenograms of the chest were taken and the Mantoux test (using the old-tuberculin of a dilution of 1:2000) was performed. The positive Mantoux test was that which showed an area of redness of more than 10×10 mm. 48 hours after the injection.

The children who had once been treated or were under way of treatment by any of antituberculous drugs, further those who were vaccinated with BCG, were excluded from this investigation.

2) The preparation of the antisera against tubercle bacilli.

Immune Serum C. Immune Serum C was prepared in the same way[1] as Immune Serum A except for the use of the human erythrocytes of Type AB instead of rabbit erythrocytes. Immune Serum C was of a titer of a dilution of 1:2560 against the human erythrocytes of Type O sensitized with the old tuberculin of a dilution up to 1:15, and the Serum also was of a titer of 1:1280 against the human erythrocytes sensitized with the 0.3 cm% solution of tuberculin-polysaccharide. Serum C caused the agglutination of the human erythrocytes of Type O, which had been sensitized with the old tuberculin of a dilution up to 1:10240, or sensitized with the 0.18 mg% solution of tuberculin-polysaccharide.

Immune Serum D. Immune Serum D was prepared in the same way[1] as Immune Serum B except for the use of Immune Serum C instead of Immune Serum A. Immune Serum D should not cause the agglutination with the human erythrocytes of Type O sensitized with the old tuberculin of a dilution up to 1:15.

For the conservation of Sera C and D, they were dried in vacuum, sealed in

ampules and kept at 0°C, and they were, just before use, dissolved in 0.15 *M* phosphate buffer of pH 7 so as to be of the same concentration as the original sera. By means of this conservation procedure, no changes in the titers of the sera were observed even in the lapse of 6 months after the sealing.

3) Sensitization of human erythrocytes with old tuberculin or with tuberculin-polysaccharide.

This was performed in the same way as reported in a preceding paper of ours[2] except for the use of the human erythrocytes of Type O instead of rabbit erythrocytes.

4) The preparation of tuberculin-polysaccharide.[1]

5) The method for the estimation of the antibodies against tuberculin and the antibody against the tuberculin-polysaccharide in the sera from children.

Middlebrook-Dubos' method[4] was adopted with a slight modification[1]. Blood was taken from the veins of patients, and serum was separated and inactivated at 56°C for 30 minutes. The inactivated serum was diluted with 0.15 *M* phosphate buffer of pH 7 to get a series of titers 10, 20, 40, 80, 160, and 320 times. Two drops of each of the diluted sera were put on the glass with 6 hollows, 26 mm. in diameter and 5 mm. in depth, and two drops of the suspension of the human erythrocytes of Type O sensitized with the old tuberculin of a dilution up to 1:15 were added. The contents were mixed and kept at 37°C for five hours. The highest titer of the dilution of the sera, which caused the agglutination of the erythrocytes, was read macroscopically.

For the estimation of the antibody against the tuberculin-polysaccharide in the serum, the same procedures as above mentioned were performed except for the use of the 30.4 mg% solution of tuberculin-polysaccharide instead of old tuberculin.

6) The method for the detection of "the erythrocytes sensitized with tuberculin-polysaccharide" in the circulating blood.

The same procedures as described in a preceding paper of ours[1] were carried out except for the use of Immune Sera C and D instead of Sera A and B respectively.

The erythrocytes were separated by centrifugation from the citrated blood taken from the veins or the ear lobes of children, and washed three times with 0.15 *M* phosphate buffer of pH 7. After centrifugation, 0.02 cc. (or 0.01 cc.) of the erythrocyte deposit was put into a test tube which contained 4.0 cc. (or 2.0 cc.) of the phosphate buffer (—this is "the suspension of the erythrocytes"). Immune Serum C was diluted with the phosphate buffer to get a series of titers 1, 2, 4 and 8 times, and two drops of each of the diluted sera were put in the glass plate with hollows, and one drop of "the suspension of the erythrocytes" was added, then the contents were mixed, and kept at 37°C for five hours. The highest titer of the dilution of the sera, which caused the agglutination of the erythrocytes, was read macroscopically. As a control test, the same procedures were above mentioned were carried out simultaneously, except for the use of the Immune Serum D instead of Immune Serum C.

Results and Discussion

Erythrocytes sensitized with tuberculin-polysaccharide were detected in the circulating blood of 89 cases (32.0%) out of 278 patients under 13 years of age.

(i) Relation among the sensitized erythrocytes, familial contact with tuberculous patient, X-ray findings of the chest and Mantoux test according to the age groups.

The results were given in Tables I and II, and in Figs. 1–3 (Cf. Tables I–II, and Figs. 1–3).

Table I

Relation among Sensitized Erythrocytes, Familial Contact with Tuberculous Patient, X-ray Findings of Chest and Mantoux Test according to Age Group

Groups of age (years)	No. oi cases	Cases in which the erythrocytes sensitized with tuberculin were identified fn circulating blood				Cases in which the erythrocytes sensitized with tuberculin were not identified in circulating blood			
		No. of cases	Cases with familial contact with tuberculous patient	Case with the positive Mantoux test	Case with the pathological findings in X-ray	No. of cases	Cases with familial contact with tuberculous patient	Cases with the positive Mantoux test	Cases with the pathological findings in X-ray
0– 3	121	47 (38.8%)	26 (55.2%)	15 (31.9%)	21 (44.6%)	74	11 (14.8%)	14 (18.9%)	16 (21.6%)
3– 7	80	27 (33.7%)	7 (25.9%)	10 (37.0%)	18 (66.6%)	53	10 (18.8%)	18 (33.9%)	26 (49.0%)
7–13	77	15 (19.4%)	3 (20.0%)	11 (73.3%)	12 (80.0%)	62	14 (23.2%)	47 (75.8%)	29 (46.7%)
	278								

The cases with erythrocytes sensitized with tuberculin-polysaccharide were found in 47 cases (38.8%) out of 121 cases under 3 years of age, in 27 cases (33.7%) out of 80 cases of the age of from 3 to 7 years, and in 15 cases (19.4%) out of 77 cases of the age of from 7 to 13 years. Among these three figures of percentage of the cases with the sensitized erythrocytes the former two were higher with a statistical significance than the last (Cf. Table I).

The percentage of the cases with a history of contact with tuberculous patient in the family was higher with a statistical significance in the cases with the sensitized erythrocytes than in those without the sensitized erythro-

TABLE II

Antibody against Tuberculin-Polysaccharide and Middlebrook-Dubos' Reaction in Cases in which Erythrocytes Sentisized with Tuberculin were detected in Circulating Blood

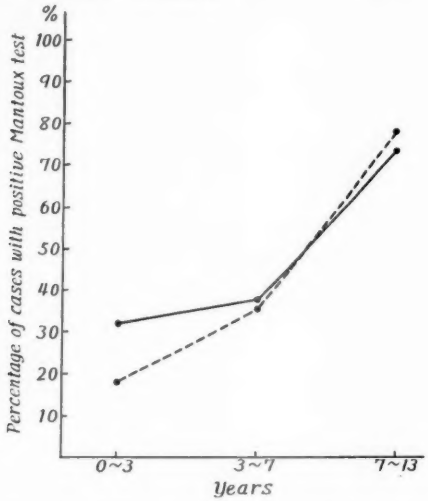
Group of age (years)	No. of case	Antibody against tuberculin-polpsaccharide in serum							Middlebrook-Dubos' reaction of serum						
		Negative	Positive (Dilution of antiserum)						Negative	Positive (Dilution of antiserum)					
			10	20	40	80	160	320		10	20	40	80	160	320
- 3	47	47	0	0	0	0	0	0	30	10	5	1	1	0	0
										17 cases (36%)					
3- 7	27	27	0	0	0	0	0	0	9	6	9	2	2	0	0
										19 cases (70%)					
7-13	15	15	0	0	0	0	0	0	3	6	2	1	2	0	1
										12 cases (80%)					

cytes in the children under 3 years of age (Cf. Fig. 1).

On the other hand, the following tendency was worthy of note: 1) the percentage of the cases with the positive Mantoux test was higher in the cases with sensitized erythrocytes than in those without them in the children under three years of age (Cf. Fig. 2), and 2) in all the age groups, the percentage of the cases with the pathological X-ray findings was higher in the cases with the sensitized erythrocytes than in those without them (Cf. Fig. 3).

In Table I it was to be noted that, out of the cases with erythrocytes sensitized with tuberculin, the cases with positive Mantoux reaction, using old tuberculin of a dilution of 1 : 2000, were found in only 31–37% of the subjects under 7 years of age. In other words, 69–63% of the children with erythrocytes sensitized with tuberculin were those in which "tuberculous infection" either was only suspect or was not suspected of at all due to the negative Mantoux reaction. Even though tuberculin reaction would remain negative, an evidence for tuberculous infection could be definitely demonstrated by positive culture of tubercle bacilli in materials obtained from the patients in question, and such cases are encountered not infrequently especially in infants and children. According to Kumagai and Mikami,[7] tuberculin reaction, using old tuberculin of a dilution of 1:1000, remained negative in 6 cases out of 11 cases in which positive culture of tubercle bacilli was obtained in gastric washings, pharyngeal swabs or faces of children under 6 years of age. In view of the report above quoted, it may be not amazing that, in our present investigation, tuberculin reaction was positive in only 25 cases (33.7%) out of 74 cases

Fig. 1. Cases with or without the sensitized erythrocytes and their relation to familial contact with tuberculous patient.



●——● Cases in which the erythrocytes sensitized with tuberculin were identified in the circulating blood.

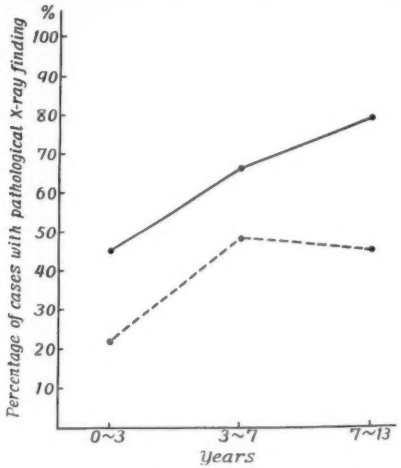
●- - -● Cases in which the erythrocytes sensitized with tuberculin were not identified in the circulating blood.

—children under 7 years of age—in which the erythrocytes sensitized with tuberculin-polysaccharide were identified in their circulating blood. The detection of the erythrocytes sensitized with tuberculin in the circulating blood will, therefore, be valuable for the diagnosis of tuberculous infection in the cases with negative Mantoux reaction[5),7)] and negative roentgenograms.

(ii) Relation among occurrence of the sensitized erythrocytes, Middlebrook-Dubos' reaction, and the antibody against the tuberculin-polysaccharide in the serum.

Of all the cases in which the erythrocytes sensitized with tuberculin-polysaccharide were identified in the circulating blood, there was not a single case in which the antibody against tuberculin-polysaccharide was ever found (Cf. Table II). This result showed a good accordance with those reported in a preceding paper of ours,[1)] which demonstrated that, when rabbits were infected with tubercle bacilli, the erythrocytes sensitized with tuberculin-polysaccharide were found in the very early stage of infection, and they had disappeared from the circulating blood as soon as the antibody against the tuberculin-polysaccharide came to be developed

Fig. 2. Cases in which the erythrocytes sensitized with tuberculin were identified in the circulating blood.



●——● Cases in which the erythrocytes sensitized with tuberculin were identified in the circulating blood.

●- - -● Cases in which the erythrocytes sensitized with tuberculin were not identified in the circulating blood.

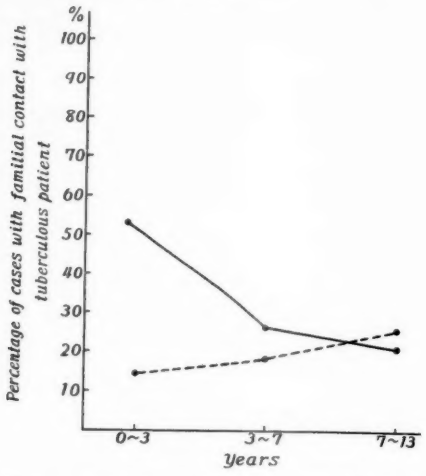
in the serum.

On the other hand, positive Middlebrook-Dubos' reaction was found among the cases with the erythrocytes sensitized with tuberculin (Cf. Table II). This finding suggested that the positivity of Middlebrook-Dubos' reaction, using old tuberculin for sensitization of human red cells, showed not only the existence of antibody against tuberculin-polysaccharide, but also the existence of other antibodies which, though not derived from tubercle bacilli themselves, caused an agglutination of the erythrocytes sensitized with tuberculin.[3),6)]

The identification of the erythrocytes sensitized with tuberculin-polysaccharide in circulating blood may be one of valuable methods for the diagnosis of tuberculous infection of children under 7 years of age, and we believe that it will be one of the first clues to the detection of tuberculous infection in children with negative reaction to the Mantoux test and other negative findings.

As to the influence of treatment with antituberculous drugs upon the erythrocytes sensitized with tuberculin-polysaccharide, upon the Mantoux reaction and upon the antibody against tuberculin-polysaccharide in serum, the results will be reported in our next paper.[8)]

Fig. 3. Cases with or without the sensitized erythrocytes and their relation to pathological X-ray finding of the chest.



●——● Cases in which the erythrocytes sensitized with tuberculin were identified in the circulating blood.

●- - -● Cases in which the erythrocytes sensitized with tuberculin were not identified in the circulating blood.

Summary

Erythrocytes sensitized with tuberculin-polysaccharide were identified in the circulating blood from 89 cases (32.0%) out of 278 patients under 13 years of age.

The incidence of the cases with the sensitized erythrocytes was higher below 7 years than above 7 years of age.

Antibody against tuberculin-polysaccharide in serum was not detected in any of the cases with the sensitized erythrocytes.

Positive Mantoux reaction, tried with the old tuberculin of a dilution of 1:2000, was found in only 33.7% out of 74 cases with the sensitized erythrocytes under 7 years of age.

Conclusions

The identification of the erythrocytes with tuberculin-polysaccharide in circulating blood will be one of valuable methods for the diagnosis of tuberculous infection of children under 7 years of age, especially in cases with negative tuberculin reaction and with negative roentgenograms.

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The Tohoku Journal of Experimental Medicine, Vol. 68, No. 2, 1958

The Effect of Magnesium Chloride on the Adrenalino-Secretory Action of Potassium Chloride

By

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(Received for publication, March 17, 1958)

Evidence concerning the antagonistic actions of K and Mg ions on the adrenaline secretion of the adrenal was given by Hazard and Wurmser.[1] They observed in dogs that the elevation of blood pressure evoked by intravenous injection of KCl was prevented by $MgCl_2$ and assumed that this was attributable to the antagonistic effect of $MgCl_2$ on the adrenalino-secretory action of KCl.

Their experiment seems to be sufficiently reasonable to know the antagonistic actions of two ions on the cardio-vascular system at least, but we feel there is a need to investigate quantitatively this problem because the blood pressure response is not a specific indicator for the adrenaline secretion of the adrenal.

Thus, the present study was undertaken to elucidate quantitatively whether the accelerated adrenaline secretion causable by KCl is suppressed by $MgCl_2$.

Experimental

Methods

Dogs, anesthetized by intravenous injection of Evipan-sodium, were used. The operation for collecting the adrenal venous blood was performed through the lumbar route with almost the same technique as that of Satake *et al.*[2] The adrenaline content of the blood specimens was determined chemically by the arseno-molybdic acid method of Bloor and Bullen.[3] In most experiments the blood pressure was recorded by the mercury manometer connected with the femoral artery.

KCl in a dose of 30–33 mg. per kg. of body weight was injected intravenously in 15 seconds. Twenty to 30 minutes later, a mixture of KCl in the same amount as before with $MgCl_2$ in an amount of 10 mg. per kg. was again injected in-

travenously in 15 seconds.

Thus, the adrenaline secretion rates after the first and the second injections were estimated and compared with each other. Adrenaline (Merck), dissolved in 1/10 *N* hydrochloric acid solution, was taken as the reference standard.

Results

The data obtained from four experiments are epitomized in Table I.

TABLE I

Effect of $MgCl_2$ on the Augmented Adrenaline Secretion Causable by KCl

No. of exp. Body weight and Sex	Adrenaline secretion rate (μg. per kg. per minute)					
	Injection of KCl			Injection of KCl plus $MgCl_2$		
	Before	After		Before	After	
		0–60″	60″–120″		0–60″	60″–120″
1) 8.2 kg. ♂	*	0.09	0.02	0.005	0.01	0.006
2) 11.1 kg. ♂	*	0.31	0.04	0.008	0.009	*
3) 9.9 kg. ♀	0.02	0.11	0.02	0.02	0.03	0.01
4) 17.0 kg. ♂	*	0.38	0.06	0.02	0.10	*

* Immeasurably small

In Exp. 1 the adrenaline content of the blood sample collected before KCl injection was too small to be quantified. After KCl a definite increase in the adrenaline secretion rate was found. In the first 60-second period after the start of injection it was determined as 0.09 μg. per kg. per minute. With the specimen, which was collected during the second 60-second period, the rate of secretion was determined as 0.02 μg.

Thirty minutes later, the rate of secretion was estimated as 0.005 μg. per kg. per minute. Then the mixture of KCl with $MgCl_2$ was injected. After injection the rate of secretion did not show any definite alteration.

In Exp. 2, the basal rate of adrenaline secretion at the beginning of experiment was immeasurably small. On receiving KCl it was increased. With the specimen collected during the first 60-second period, it was found to be 0.31 μg. per kg. per minute. In the second 60-second period it was 0.04 μg.

Twenty-five minutes later, the rate of secretion was 0.008 μg. per kg. per minute. The mixture of KCl with $MgCl_2$ was then injected. After the application the rate of adrenaline secretion was determined as 0.09 μg. and immeasurably small rate at the first and the second 60-second period, respectively.

In Exp. 3 the initial adrenaline secretion rate was 0.02 μg. per kg. per minute. By the administration of KCl, the increase in the adrenaline secretion rate up to 0.11 μg. was elicited, which was found in the first 60-second period. In the second 60-second period the rate of secretion was 0.02 μg.

Twenty minutes later, the adrenaline secretion rate of the adrenal was 0.02 μg. per kg. per minute. Then the injection of KCl plus $MgCl_2$ was made. By the injection the rate of secretion was not increased definitely.

In Exp. 4, the adrenaline secretion rate after KCl injection was found to be 0.38 μg. per kg. per minute in the first 60-second period against immeasurably small rate before injection. In the next 60-second period it was 0.06 μg.

Twenty-two minutes later, the adrenaline secretion rate was 0.02 μg. per kg. per minute. By the injection of KCl plus $MgCl_2$ its value was somewhat increased, 0.10 μg. being estimated in the first 60-second period.

From the above results, it was clarified quantitatively that the augmentation of the adrenaline secretion causable by KCl was suppressed or nullified by $MgCl_2$.

In most cases the blood pressure fell abruptly after KCl injection and at its lowest level a bradycardia was observed. Then the blood pressure rose again step by step and resumed the initial level. After the injection of the mixture of KCl with $MgCl_2$, the blood pressure fell in most cases slightly. Soon, it began to rise and reached the pre-injection level.

Summary

Four dogs, anesthetized with Evipan-sodium, were experimented on. The adrenal venous blood specimens were collected through the lumbar route and were analysed for adrenaline by the chemical method of Bloor and Bullen.

While the administration of KCl in a dose of 30–33 mg. per kg. caused a definite increase in the adrenaline secretion, the injection of KCl in the same dose as before plus $MgCl_2$ in a dose of 10 mg. per kg. produced no increase or only a small increase in the adrenaline secretion.

From these experimental results, it is concluded that $MgCl_2$ is capable of antagonizing the accelerating action of KCl on the adrenaline secretion.

We should like to record our gratitude to Prof. T. Suzuki for suggesting the problem.

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The Tohoku Journal of Experimental Medicine, Vol. 68, No. 2, 1958

Molisch-positive Mucopolysaccharides of Gastric Cancers as Compared with the Corresponding Components of Gastric Mucosae

Second Report

On MPSs (Mucopolysaccharides) I*

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(Received for publication, March 20, 1958)

The physical, chemical and biological properties of MPSs I from gastric cancers and non-cancerous gastric mucosae of group A, B or O, whose preparation procedure was described in the First Report,[1] will be accounted for. The findings detailed in the experimental part below may be outlined :—1) The six different MPSs I showed the same electrophoretic mobilities in an acid and an alkaline buffer, regardless whether the material of preparation was cancerous or non-cancerous, and whether it belonged to group A, B or O. 2) Group A and B MPSs I from the non-cancerous gastric mucosae are very strongly group A or B active, but those from gastric cancers are much weaker. However, group O activities of the group O MPSs I, cancerous and non-cancerous, are both intensive and that nearly to equal extents. 3) All MPSs I were found to contain galactosamine, glucosamine, galactose, L-fucose and sialic acid as sugars, and aspartic and glutamic acids, serine, glycine, lysine, histidine, arginine, threonine, alanine, proline, valine and/or methionine, and leucine and/or isoleucine and/or phenylalanine as amino acids by paper-chromatography. 4) As to the quantitative composition, i) there are almost no differences among the mucopolysaccharides in content of nitrogen, total hexosamine, galactose and L-fucose and in iodine use, ii) the glucosamine/galactosamine ratio varies distinctly from group to group in the non-cancerous mucopolysaccharides but does not in the cancerous, and iii) sialic acid amounts more in the cancerous substances than in the non-cancerous, although it

* 215th report of Masamune and co-workers' "Biochemical Studies on Carbohydrates." 4th report on "Chemical Nature of Toxohormone (Nakahara)." A part was read before the XVIth General Meeting of the Japanese Cancer Association, Osaka, October 30-31, 1957.

is generally scarce. 5) The non-cancerous mucopolysaccharides rotate polarized sodium-light to the left in different grades. On the contrary, the cancerous substances resemble one another in optical rotation, all being more levorotatory than the non-cancerous. 6) The molecular weight of the cancerous group A mucopolysaccharide is lower than that of the non-cancerous mucopolysaccharide of the same group, as calculated from their intrinsic viscosities and sedimentation constants. 7) All MPSs I behave as toxohormones, though weak.

The facts revealed here will be discussed in a later article together with those with respect to MPSs IV.

Experimental

Electrophoretic examination. As shown in Fig. 1, the non-cancerous MPSs I gave single symmetric boundaries in an acidic as well as an alkaline buffer. The cancerous MPSs I gave single boundaries which, however, were not strictly symmetrical, suggesting that they are only essentially homogeneous. The six substances emigrate with the same mobilities in either of the buffers, and mixtures of two picked out of them showed also single boundaries.

Blood group potency. It was measured by the glass plate method described by Masamune *et al.*[2] The results are embodied in Table I. The non-cancerous group A and B MPSs I are strongly group A or B active, but the cancerous are weak in so far as these activities are concerned. On the contrary, O group activities of the latter MPSs I are as strong as those of the former. The cancerous O substance nearly compares with the non-cancerous in group O activity.

Solubility. The six mucopolysaccharides are similar to one another in this respect. They are soluble in water of any pH value, 90% (v/v) phenol, and the saturated $(NH_4)_2SO_4$ (room temperature), almost insoluble in the saturated Na_2SO_4 at 33°C, and insoluble in glacial acetic acid, ethanol, acetone and ether. They are not precipitated from water with lead acetate, cupric chloride, barium acetate, baryta and trichloroacetic acid.

Test-tube tests. The substances behaved alike in the following qualitative tests: Molisch (#), Neuberg-Saneyoshi (−), Bial (+), Dische (−), biuret (+), test for phosphorus (−), test for hydrolyzable sulfur (−), indirect Osaki-Turumi (+). The cancerous mucopolysaccharides gave slightly positive direct Osaki-Turumi reaction, while the non-cancerous only the faint reaction.

Paper partition chromatography of the components. Sugars. The procedure of Masamune and Yosizawa[3] was adapted as follows. 6 mg. of substance was hydrolyzed with 0.6 cc. of 1 *N* H_2SO_4 by heating in a sealed tube at 100°C for 5 hours. When cold, the hydrolysate was neutralized with the

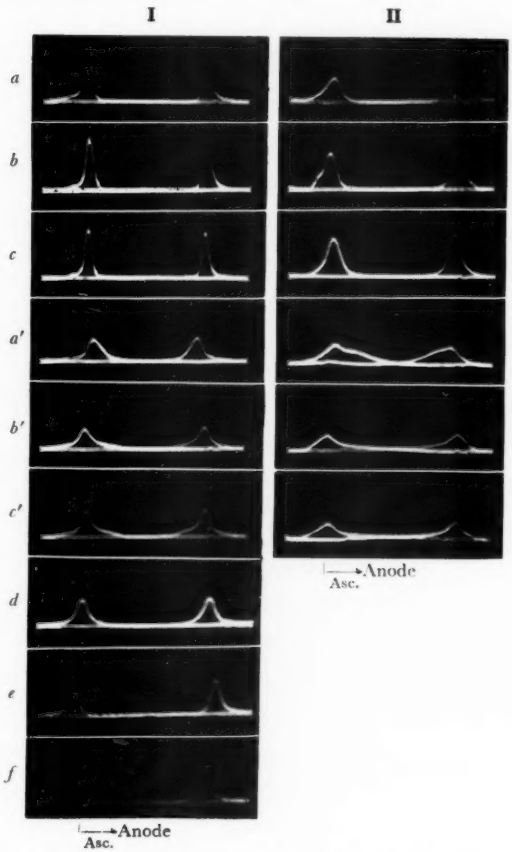

Fig. 1. Electrophoretic patterns (2/3×) of 1% solutions of the non-cancerous group A (*a*), B (*b*) and O (*c*) and cancerous group A (*a'*), B (*b'*) and O (*c'*) MPSs I in 0.1 *M* veronal-HCl buffer, pH 8.6, I = 0.1 + NaCl (0.2 *M*) (**I**) and 0.2 *M* acetate buffer, pH 4.6 (I = 0.2) (**II**), and those of mixtures of equal amounts of the cancerous group A and non-cancerous group A MPSs I (*d*), of the non-cancerous group A and O MPSs I (*e*) and of the non-cancerous group B and O MPSs I (*f*) in the veronal-HCl buffer above. Temperature 15°C; current 8 mA; exposed 120 min. after starting current.

Mobilities in $cm^2 \cdot volt^{-1} \cdot sec^{-1} \times 10^{-5}$

$$\mathbf{I}a : \frac{(-0.25)\times 0.01278\times 0.3}{0.008\times 7200} = -1.7 \qquad \mathbf{II}a : \frac{(-0.17)\times 0.00591\times 0.3}{0.008\times 7200} = -0.5$$

$$\mathbf{I}b : \frac{(-0.24)\times 0.01278\times 0.3}{0.008\times 7200} = -1.6 \qquad \mathbf{II}b : \frac{(-0.15)\times 0.00591\times 0.3}{0.008\times 7200} = -0.5$$

$$\mathbf{I}c : \frac{(-0.24)\times 0.01284\times 0.3}{0.008\times 7200} = -1.6 \qquad \mathbf{II}c : \frac{(-0.15)\times 0.00588\times 0.3}{0.008\times 7200} = -0.5$$

$$\text{I}a' : \frac{(-0.25)\times 0.01278\times 0.3}{0.008\times 7200} = -1.7 \qquad \text{II}a' : \frac{(-0.17)\times 0.00588\times 0.3}{0.008\times 7200} = -0.5$$

$$\text{I}b' : \frac{(-0.25)\times 0.01278\times 0.3}{0.008\times 7200} = -1.7 \qquad \text{II}b' : \frac{(-0.17)\times 0.00588\times 0.3}{0.008\times 7200} = -0.5$$

$$\text{I}c' : \frac{(-0.25)\times 0.01284\times 0.3}{0.008\times 7200} = -1.7 \qquad \text{II}c' : \frac{(-0.18)\times 0.00591\times 0.3}{0.008\times 7200} = -0.5$$

$$\text{I}d : \frac{(-0.27)\times 0.01280\times 0.18}{0.005\times 7200} = -1.7$$

$$\text{I}e : \frac{(-0.25)\times 0.01280\times 0.18}{0.005\times 7200} = -1.6$$

$$\text{I}f : \frac{(-0.28)\times 0.01280\times 0.18}{0.005\times 7200} = -1.8$$

saturated $Ba(OH)_2$ to pH 5.4–6.4. The precipitate was centrifuged off and washed with three 1 cc. portions of water at 60°C. The supernatant and washings were evaporated to dryness in a desiccator containing $CaCl_2$. The residue was dissolved in 0.09 cc. of water, and two 0.03 cc. portions of the solution were sampled separately on 5×60 cm. slivers of Toyo Roshi filter paper No. 2 to develop chromatograms with different solvent mixtures. Temperatures of irrigations were 23–26°C and durations of irrigations 18–24 hours. Indicator aniline hydrogen phthalate reagent of Partridge. All MPSs I proved to contain galactosamine, glucosamine, galactose and L-fucose. Glucose, mannose and pentose were not detected. In Fig. 2 are shown the sugar chromatograms of the cancerous group A MPS I. Those of the other MPSs I which closely resembled the ones reproduced are omitted.

Amino acids. 8 mg. substance was heated with 0.8 cc. of 6 *N* HCl at 100°C for 24 hours. The occurring humin was filtered off and washed with water. The filtrate and washing were evaporated to dryness at 30°C in vacuo over anhydrous calcium chloride and solid caustic soda. The evaporation was repeated several times, each residue having been dissolved with a few drops of water, until most of the hydrochloric acid was expelled. The residue finally obtained was dissolved in 0.08 cc. of water, and 0.03 cc. of the solution was applied on a 30×30 cm. square of Toyo Roshi filter paper No. 50. The primary irrigation was carried out with Solvent butanol-acetic acid-water (4:1:2 by volume) and the secondary was with Solvent 80% phenol in an ammonia-containing atmosphere. Temperatures of irrigations were 23–26°C. Durations of the primary irrigations were 8–10 hours, and those of the secondary were 10–13 hours. The indicator used was 0.2% ninhydrin in moist butanol. All of those mucopolysaccharides gave spots at the positions corresponding to aspartic and glutamic acids, serine, glycine, lysine, histidine, arginine, hexosamine, threonine, alanine, proline, valine and/or methionine, and leucine and/or isoleucine and/or phenylalanine. Fig. 3 shows the amino

TABLE I

Anti-hemagglutinative Potencies of MPSs I of Stomach Cancers and of Stomach Mucosae

Goat: normal anti-O goat serum. Anti-E: eel serum. Anti-OS: anti-OS saliva chicken serum absorbed with ABe red cells*. − no hemagglutination, ± faint hemaggl., + slight hemaggl., ++ marked hemaggl., +++ strong hemaggl.

MPS I				Dilution of the substances ($1:10^3\times$)																
Source	Blood group	Red cells	Serum	1	2	4	8	16	32	64	128	256	512	1,024	2,048	4,096	8,192	16,384	32,768	65,536	∞
Stomach cancer	A	A	α	−	−	−	−	−	−	−	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		B	β	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		O	Goat	−	−	−	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		O	Anti-E	−	−	−	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		O	Anti-OS	−	−	−	−	−	−	−	−	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	B	A	α	−	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		B	β	−	−	−	−	−	±	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		O	Goat	−	−	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		O	Anti-E	−	−	−	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		O	Anti-OS	−	−	−	−	−	−	−	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	O	A	α	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		B	β	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		O	Goat	−	−	−	−	−	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		O	Anti-E	−	−	−	−	−	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		O	Anti-OS	−	−	−	−	−	−	−	−	−	−	+	++	+++	+++	+++	+++	+++	+++	+++
Stomach mucosa	A	A	α	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	++	+++	+++
		B	β	±	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		O	Goat	−	−	−	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		O	Anti-E	−	−	−	−	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		O	Anti-OS	−	−	−	−	−	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	B	A	α	−	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		B	β	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	++	+++	+++
		O	Goat	−	−	−	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		O	Anti-E	−	−	−	−	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		O	Anti-OS	−	−	−	−	−	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	O	A	α	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		B	β	−	−	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		O	Goat	−	−	−	−	−	−	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		O	Anti-E	−	−	−	−	−	−	−	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		O	Anti-OS	−	−	−	−	−	−	−	−	−	−	−	−	++	+++	+++	+++	+++	+++	+++

* Anti-OS saliva chicken serum was diluted with 9 volumes of physiological saline, and 9.5 cc. of it was stood with 7.5 cc. of ABe red cells at 37°C for 2 hrs. and centrifuged. The supernatant was again absorbed with 5 cc. of the same red cells in a similar manner. The centrifuged supernatant here agglutinated ABe red cells at a dilution of 1:10 but not at a dilution of 1:20. It was diluted to anti-O titer 16 in advance of use.

acid chromatogram of the cancerous group A MPS I. Those of the other MPSs I are omitted, because they showed spots at the same posi-

Fig. 2

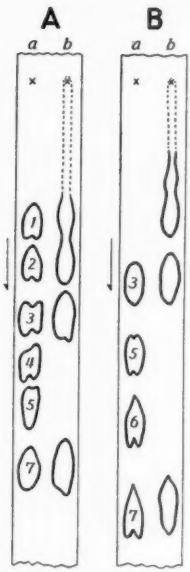

Fig. 3

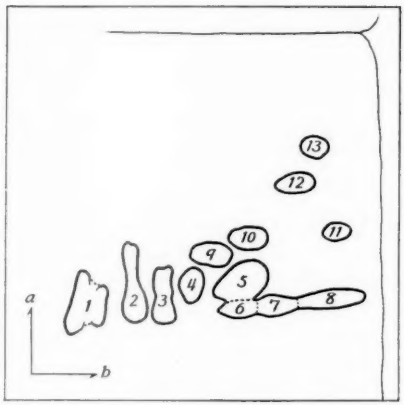

Fig. 2. Sugar paper chromatograms of the cancerous group A MPS I, developed with Solvent butanol-pyridine-water (5:3:2 by volume) (**A**) or with Solvent *n*-butylacetate-acetic acid-ethanol-water (3:2:1:1 by volume) (**B**). Temperature and duration of the irrigations were 24°±1°C and 24 hrs. respectively. Spray reagent 2% aniline phthalate in moist butanol. Reference runs were carried out, using 0.02 cc. of 1% solutions with respect to individual sugars.

a: reference run. *b*: the hydrolysate of the cancerous group A MPS I. *1* galactosamine, *2* glucosamine, *3* galactose, *4* glucose, *5* mannose, *6* xylose, *7* L-fucose.

Fig. 3. Amino acid paper chromatogram of the cancerous group A MPS I. (*a*) primary irrigation (10 hrs., 23°±1°C). (*b*) secondary irrigation (13 hrs., 23°±1°C). Spray reagent 0.2% ninhydrin in butanol saturated with water.

1 asparatic acid, *2* glutamic acid, *3* serine, *4* glycine, *5* hexosamine, *6* lysine, *7* histidine, *8* arginine, *9* threonine, *10* alanine, *11* proline, *12* valine and/or methionine, *13* leucine and/or isoleucine and/or phenylalanine.

tions.

Quantitative composition. The analytical figures are embodied in Table II. The contents of nitrogen, total hexosamine, galactose and L-fucose and iodine use as glucose show no remarkable discrepancies among the mucopolysaccharides. Sialic acid amounts more in the cancerous sub-

TABLE II

Composition of MPSs I of Stomach Cancers and of Stomach Mucosae

Analysis	In per cent						In equivalents per equivalent weight					
	Stomach cancer			Stomach mucosa			Stomach cancer			Stomach mucosa		
	Group A	B	O	Group A	B	O	Group A	B	O	Group A	B	O
N*	4.7	4.6	4.6	5.0	4.7	5.0	2.18	2.00	1.93	2.39	2.04	2.21
Total hexosamine†	30.6	33.0	32.1	33.5	32.3	34.3	1.11	1.12	1.05	1.20	1.09	1.19
$\frac{\text{Glucosamine}}{\text{Galactosamine}}$ ratio†	3.1	3.2	3.2	2.3	3.5	3.1						
Galactose‡	27.8	29.7	30.7	28.1	29.7	29.1	1.0	1.0	1.0	1.0	1.0	1.0
L-Fucose§	17.1	15.1	16.9	15.2	16.0	16.5	0.67	0.56	0.60	0.60	0.59	0.62
Sialic acid//	2.7	4.2	4.3	0.9	1.1	1.4	0.06	0.08	0.08	0.02	0.02	0.03
Ash¶	1.6	1.3	1.5	1.0	0.4	0.6						
Iodine use as glucose**	2.7	?	2.6	3.0	2.7	2.7						

* Micro Kjeldahl. † Masamune and Yosizawa[4]. ‡ Reaction PHR_2 of Masamune and Sakamoto.[5]. § A modification of Dische and Shettles[6]. // Direct Ehrlich reaction of Werner and Odin[7] ; Calcd. as $C_{11}H_{19}NO_9$. ¶ Pregl method without use of H_2SO_4. ** Macleod and Robison[8] ; Iodine use of the cancerous group B MPS I could not be determined because of shortage of material.

TABLE III

Intrinsic Viscosity of the Cancerous and Non-cancerous Group A MPSs I at 20°C

MPS I		No. of solution	C (Concentration in g/dl)	η_r (Relative viscosity)	η_{sp} (Specific viscosity)	η_{sp}/C	$[\eta]$ (Intrinsic viscosity)
Source	Blood group						
Stomach cancer	A	1	0.5158	1.223	0.223	0.432	0.404
		2	0.3683	1.157	0.157	0.426	
		3	0.2576	1.108	0.108	0.419	
		4	0.1841	1.075	0.075	0.407	
		5	0.1104	1.046	0.046	0.417	
Stomach mucosa	A	6	0.6000	1.267	0.267	0.445	0.343
		7	0.4286	1.178	0.178	0.415	
		8	0.3061	1.121	0.121	0.395	
		9	0.2187	1.083	0.083	0.380	
		10	0.1469	1.054	0.054	0.368	
		11	0.1094	1.040	0.040	0.366	

stances than in the non-cancerous. As to the glucosamine/galactosamine ratio, the cancerous substances resemble one another, although the non-cancerous differ from group to group.

Optical rotation in water. The non-cancerous mucopolysaccharides are levorotatory to various extents. However, the cancerous substances rotate sodium-light alike, all being more levorotatory than the non-cancerous.

Cancerous MPSs I

Group A $[\alpha]_D^{14}=\frac{-0.394\times 1}{0.0105\times 1}=-37.5°$

Group B $[\alpha]_D^{15}=\frac{-0.388\times 1}{0.0102\times 1}=-37.8°$

Group O $[\alpha]_D^{17}=\frac{-0.411\times 1}{0.0102\times 1}=-40.6°$

Non-cancerous MPSs I

Group A $[\alpha]_D^{14}=\frac{-0.218\times 1}{0.0111\times 1}=-19.6°$

Group B $[\alpha]_D^{15}=\frac{-0.336\times 1}{0.0119\times 1}=-28.2°$

Group O $[\alpha]_D^{17}=\frac{-0.397\times 1}{0.0115\times 1}=-34.5°$

All the solutions examined were of pH 6.8, excepting that of the cancerous group O MPS I which was of pH 6.6.

Ultraviolet absorption. A photoelectric spectrophotometer (EPB-U type, Hitachi) was used. Cell 1 cm. thick. See Fig. 4. The absorption spectra showed no distinct maxima near the wave-lengths 260 and 270 mμ, indicating the absence or presence only in trace of nucleic acids and cyclic amino acids.

Molecular axial ratio and molecular weight. Those constants were derived in the way described by Suzuki,[9] limiting the objects to the group A MPSs I, cancerous and non-cancerous. The solvent used was $M/15$ phosphate buffer of pH 7.0 (I 0.147). i) Partial specific volume. Apparent partial specific volume Va was found to be 0.618 for the cancerous mucopolysaccharide and 0.625 for the non-cancerous (Temperature 20°C). They will be assumed as partial specific volumes V.

ii) Volume intrinsic viscosity. By means of the same vicosimeter that had been used by Suzuki, the figures in Table III were obtained. By equation $\nu=100\frac{[\eta]}{V}$, where ν is volume intrinsic viscosity and $[\eta]$ is intrinsic viscosity, ν was calculated at 65.4 for the cancerous substance and at 54.9 for the non-cancerous.

iii) Axial ratio of molecule. The axial ratios, which were read from Mehl and others' f curves,[10] approximately amounted to

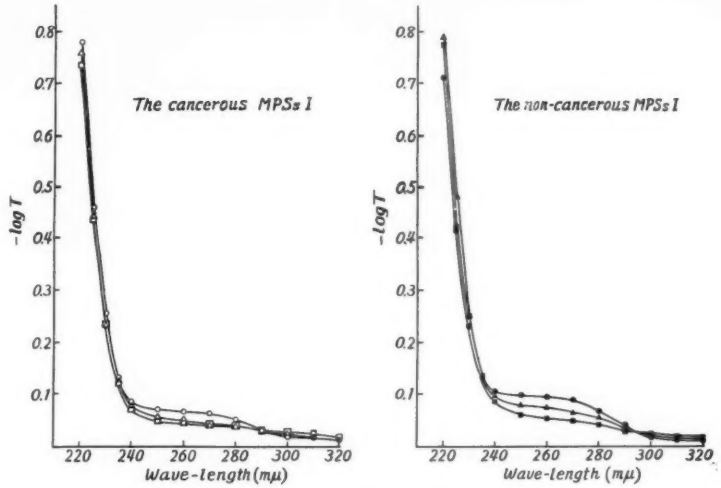

Fig. 4. Ultraviolet absorption spectra of 50 mg% solutions of the cancerous group A (—○—○—), B (—□—□—) and O (—△—△—) and non-cancerous group A (—●—●—), B (—■—■—) and O (—▲—▲—) MPSs I in water. pH values of the solutions were 6.4–6.8.

TABLE IV

Sedimentation Constants of the Cancerous and Non-cancerous Group A MPSs I in $M/15$ Phosphate Buffer, pH 7.0

MPS I		No. of solution	Concentration (g/dl)	Rotation speed (mean) (rev./min.)	Rotor temperature (mean) (°C)	x Distance of boundary from axis of rotation (cm.)		Δt (sec.)	$\Delta x = x_2 - x_1$	Sedimentation constant $\times 10^{13}$	
Source	Blood group					x_1 (initial)*	x_2 (after Δt)			Measured value	$S_{20,w}$
Stomach cancer	A	1	0.26	58,000	6.4	5.910	6.026	1976	0.116	2.66	4.06
		2	0.52	58,100	5.2	5.920	6.026	1976	0.106	2.43	3.85
		3	1.01	58,500	6.6	6.026	6.133	1964	0.107	2.39	3.55
Stomach mucosa	A	4	0.22	58,000	9.6	6.000	6.154	1980	0.154	3.47	4.80
		5	0.44	57,000	7.7	5.926	6.066	2016	0.140	3.25	4.76
		6	1.03	57,700	7.4	6.066	6.206	1984	0.140	3.15	4.66

* Means x when photographed at first.

28/1 for the cancerous substance as rods,
96/1 for the same substance as disks,
25/1 for the non-cancerous as rods, and

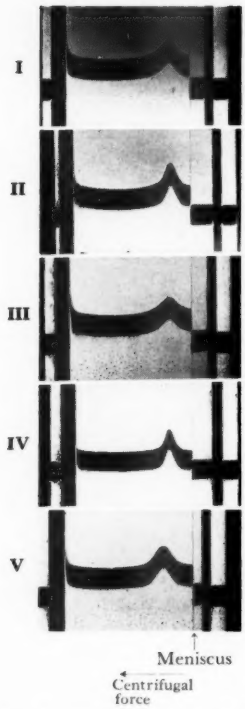

Fig. 5. Sedimentation diagrams (2.3×) of solutions of MPSs I in *M*/15 phosphate buffer of pH 7.0 (I 0.147). **I**) Solution No. 3 in Table IV of the cancerous group A MPS I; 42 minutes after reaching full speed; bar angle 70°. **II**) Solution No. 6 in Table IV of the non-cancerous group A MPS I; 40 minutes after reaching full speed; bar angle 70°. **III**) A 1% solution of the cancerous group O MPS I; 44 minutes after reaching full speed 59,730 rev./min.; rotor temperature 12.8°C; bar angle 70°. **IV**) A 1.3% solution of the non-cancerous group B MPS I; 47 minutes after reaching full speed 59,770 rev./min.; rotor temperature 8.9°C; bar angle 80°. **V**) A 1.1% solution of the non-cancerous group O MPS I; 55 minutes after reaching full speed 59,730 rev./min.; rotor temperature 17.7°C; bar angle 70°.

80/1 for the same substance as disks.

iv) Sedimentation constant (The writer is indebted to Mr. Y. Amano in the Bacteriological Department). Ultracentrifugation was effected in a Spinco ultracentrifuge Model-E. The sedimentation constants observed $S_{20,w}$ were plotted against concentrations and extrapolated to 0 concentration to obtain that of the ideal solution, giving value 4.20×10^{-13} for the cancerous substance and value 4.84×10^{-13} for the non-cancerous. See

Table IV and Fig. 5.

v) Molecular weight. It was calculated from the axial ratio and sedimentation constant by equation:

$$M=2.45\times10^{22}\times V_{20}{}^{1/2}\ [(f/f_o)S_{20,w}/(1-0.9982V_{20})]^{3/2}$$

where M is molecular weight, V_{20} is partial specific volume at 20°C, f/f_o is frictional ratio, and $S_{20,w}$ is sedimentation constant of the ideal solution. M calculated[11] was

7.7×10^4 for the cancerous substance as rods ($f/f_o=2.29$),
11.1×10^4 for the same substance as disks ($f/f_o=2.93$),
8.9×10^4 for the non-cancerous as rods ($f/f_o=2.18$), and
12.8×10^4 for the same substance as disks ($f/f_o=2.77$).

(f/f_o values were calculated neglecting the effect of hydration.)

Anemia-inducing potency (*KIK activity*). It was measured as by Masamune and Kawasaki.[12] A mixture of equal amounts of the cancerous A, B and O mucopolysaccharides and the non-cancerous B mucopolysaccharide were examined. They were injected intravenously into rabbits in dosage of 3 mg. per kg. body weight. As is shown in Table V, the decreases of number of erythrocytes here did not reach 10%, notwithstanding that the KIK factors hitherto investigated in this laboratory are effective in dosage of even 0.3 mg. or less per kg. body weight.

TABLE V

Anemia-inducing Potency of a Mixture of the Cancerous Group A, B and O MPSs I and the Non-cancerous Group B MPS I

No. of assay	Rabbit			Material injected	Dosis per kg. body wt. (mg.)	Number of red cells ($\times10^4$, per cmm.)						
	No.	Body wt. (kg.)	Sex			Before injection	Time after injection (hrs.) 1	2	3	4	5	Maximum decrease (%)
1	1	2.7	♂	A mixture of equal amounts of the canc. group A, B and O MPSs I	3	671	667	658	658	**650**	670	3
2	2	2.7	♂		"	694	704	**667**	669	716	697	4
3	3	2.9	♀		"	615	606	625	**593**	597	599	4
4	4	2.6	♀	Non-canc. group B MPS I	3	550	544	**538**	545	549	563	2
5	5	2.7	♀		"	578	560	561	567	557	**555**	4
6	6	2.2	♂		"	512	525	530	551	537	530	

Toxohormone activity. It was determined as described by Masamune *et al.*[13] As shown in Table VIA, the cancerous group A and non-cancerous group A, B and O MPSs I, which were examined, depressed liver catalase activity, when 5 mg. amounts of them were intraperitoneally injected

TABLE VIA

Liver Catalase Depression *in vivo* by the Cancerous Group A MPS I and Non-cancerous MPSs I

The average volume of O_2 evolved for each dosis in each test-set is expressed in per cent of the corresponding volume in the blank control.

No. of test-set	No. of mice used	Amount of the substances injected (mg.)											
		Canc. group A MPS I			Non-canc. MPS I								
					Group A			B			O		
		5	3	1	5	3	1	5	3	1	5	3	1
1	9	72.2		88.1									
2	6			94.1									
3	9				68.9		74.4						
4	6						72.1						
5	12							73.8	72.3	88.1			
6	12							72.2	81.6	98.7			
7	12							62.0	83.0	91.0			
8	6							70.3					
9	12										55.4	65.1	78.8
10	6												73.7

TABLE VIB

Effect *in vitro* on Liver Catalase of the Non-cancerous Group B MPS I

The liver homogenatss incubated with 5 mg. of the substance to follow Masamune *et al.*[12]

No. of mouse	Liver catalase activity (oxygen evolved in cc.)		
	Control I (No incubation)	Control II (Incubated without the substance)	Main (Incubated with the substance)
101	8.6	5.3	5.4
102	12.6	8.1	9.0
103	11.4	7.6	8.7
Average	10.9	7.0	7.7 (110% of Control II value)

into mice. Of the substances above, the non-cancerous group B substance was further subjected to the assays of *in vitro* effect on liver catalase and of *in vivo* effect on kidney- and blood-catalase (Table VIB and VIC). It proved innert in these points.

TABLE VIC

Effect *in vivo* on Kidney- and Blood-catalase of the Non-cancerous Group B MPS I

5 mg. amounts of the substance were injected into mice. Liver catalase activities were determined for comparison.

No. of mouse	Kidney	Blood	Liver
Oxygen evolved (cc.) in blank experiments			
104	4.0	0.9	12.5
105	3.8	1.7	9.9
106	4.8	3.0	14.0
Average	4.2	1.9	12.1
Oxygen evolved (cc.) in main experiments			
107	3.9	2.5	9.2
108	4.0	1.8	6.8
109	5.1	2.2	9.6
Average (% of the control value)	4.3 (102)	2.2 (116)	8.5 (70)

The cancerous group B and O MPSs I are analogized to be also weakly toxohormone active.

SUMMARY

1. Group mucopolysaccharides MPSs I, cancerous and non-cancerous, in the First Report were examined physically, chemically and biologically.

2. Noticeable findings will be enumerated.

i. The cancerous group A and B substances are very weak in group A or B activity, in contrast with the corresponding non-cancerous group substances.

ii. The cancerous mucopolysaccharides differ in composition from the non-cancerous, the sialic acid contents of the former being higher than those of the latter and the glucosamine/galactosamine ratio remaining the same irrespective of blood group in the former but varying from group to group in the latter.

iii. The cancerous substances rotate sodium-light in resembling grades and that more to the left than the non-cancerous, while the non-cancerous are differently levorotatory depending on blood group.

iv. The molecular weight of the cancerous group A mucopolysaccharide is lower than that of the non-cancerous substance of the same blood group.

v. MPSs I are toxohormone active, though weak.

Through the Grant Committee for Scientific Researches the Ministry of Education gave a grant in aid to us, which is gratefully acknowledged. H. Masamune.

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The Tohoku Journal of Experimental Medicine, Vol. 68, No. 2, 1958

Der Einfluss des Eserins auf die adrenalinsekretionsfördernde Wirkung des Carbaminoylcholins

Von

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(Received for publication, March 23, 1958)

In der vorstehenden Forschung[1] über die adrenalinsekretionsfördernde Wirkung von Carbaminolylcholin wurde die Beobachtung gemacht, daß Carbaminoylcholin stärker als Acetylcholin wirkt. So haben wir denn an einer Reihe von Hunden die im folgenden mitgeteilten Versuche vorgenommen, um zu wissen, ob diese Wirkung von Carbaminoylcholin wie beim Acetylcholin durch die Verabreichung des Eserins verstärkt werden kann.

Beschreibung der Versuche

Methodik

Bei den Versuchen wurden 4 Hunde von 9,3–13,0 kg. Gewicht verwandt, und als Narkotikum wurde Evipan-natrium in jedem Fall benützt.

Das Nebennierenvenenblut wurde mit Hilfe der Lumbalroutemethode von Satake und anderen[2] gesammelt, und dann auf seinen Gehalt an Adrenalin mittels der kolorimetrischen Methode von Bloor und Bullen[3] gemessen.

Carbaminoylcholinchlorid (Merck) sowie Acetylcholinchlorid (Hoffmann La Roche) wurden in die V. saphena im Verlauf von 15 Sekunden eingespritzt, und zwar in Dosen von 0,3–0,4 mg. pro kg. Körpergewicht. Eserinum salicyl. (Merck) wurde in einer Dosis von 0,3 mg. pro kg. intravenös in 30 bis 60 Sekunden injiziert. Vor Versuchsbeginn sowie einige Minuten vor der Injektion des Eserins wurde 1 mg. Atropinum sulfur. pro kg. intravenös gegeben.

Versuchsergebnisse

Die Einzelheiten der Versuchsergebnisse sind in der Tabelle zusammengestellt.

Beim Exp. 1 wurde zuerst Acetylcholin in einer Dosis von 0,3 mg. pro kg. injiziert, aber nach der Injektion ließ sich keine Vermehrung von Adrenalinsekretion mehr nachweisen. Etwa 13 Minuten später wurde Carbaminoylcholin in gleicher Dosis eingespritzt. Gleich nach der Injektion war die Erhöhung der Adrenalinsekretionsgeschwindigkeit bemerkbar; sie betrug 0,21 γg. pro kg. und Minute. Dann ließ die Sekretionsgeschwindigkeit schnell nach.

TABLE I

Einfluss des Eserins auf die adrenalinsekretionsfördernden Wirkungen des Carbaminoylcholins und des Acetylcholins

Nr. des Versuches	Körpergewicht und Geschlecht	Zeit	Nr. der Probe	Blutfluss (ccm) pro kg und Minute	Adrenalinmenge (γg) in 1 ccm	Adrenalinsekretion (γg) pro kg und Minute	Körpertemperatur (°C)	Zimmertemperatur (°C)
Exp. 1.	9,3 kg ♀	2:17 p.m.	Atropin (1 mg pro kg) intravenös injiziert.					
		2:23	1	0,46	*			
			2	0,46	*		38,1	13,5
		2:32	Acetylcholin (0.3 mg pro kg) intravenös injiziert.					
		0′—1′	3	0,45	*			
		1′—2′	4	0,38	*			
		2:40	5	0,37	*			
		2:45	Carbaminoylcholin (0,3 mg pro kg) intravenös injiziert.					
		0′—1′	6	0,63	0,33	0,21		
		1′—2′	7	0,60	0,06	0,04	37,6	13,5
		3:16	Atropin (1 mg pro kg) intravenös injiziert.					
		3:18	Eserin (0,3 mg pro kg) intravenös injiziert.					
		3:21	8	0,46	*			
		3:26	Carbaminoylcholin (0,3 mg pro kg) intravenös injiziert.					
		0′—1′	9	0,52	0,39	0,20		
		1′—2′	10	0,53	*		37,5	14
		3:32	11	0,46	*			
		3:40	Acetylcholin (0,3 mg pro kg) intravenös injiziert.					
		0′—1′	12	0,80	1,4	1,1		
		1′—2′	13	0,46	*			
		3:50					37,0	14
Exp. 2.	12,0 kg ♂	2:43 p.m.	1	0,47	0,05	0,02	37,4	15,5
		2:52	Atropin (1 mg pro kg) intravenös injiziert.					
		2:55	2	0,38	0,08	0,03		
		3:05	Carbaminoylcholin (0,4 mg pro kg) intravenös injiziert.					
		0′—1′	3	0,48	2,2	1,1		
		1′—2′	4	0,45	0,16	0,07		
		3:47	5	0,42	0,11	0,05	36,5	15,5
		3:54	Acetylcholin (0,4 mg pro kg) intravenös injiziert.					
		0′—1′	6	0,35	0,16	0,06		
		1′—2′	7	0,37	0,15	0,06		
		4:20	Atropin (1 mg pro kg) intravenös injiziert.					
		4:25	Eserin (0,3 mg pro kg) intravenös injiziert.					
		4:30	8	0,40	0,09	0,04	36,5	15,5
		4:39	Acetylcholin (0,4 mg pro kg) intravenös injiziert.					
		0′—1′	9	0,55	3,2	1,8		
		1′—2′	10	0,45	0,26	0,12		
		4:58	11	0,27	0,10	0,03		
		5:18	Carbaminoylcholin (0,4 mg pro kg) intravenös injiziert.					
		0′—1′	12	0,34	3,4	1,2		
		1′—2′	13	0,28	0,13	0,04		

Nr. des Versuches	Körpergewicht und Geschlecht	Zeit	Nr. der Probe	Blutfluss (ccm) pro kg und Minute	Adrenalinmenge (μg) in 1 ccm	Adrenalinsekretion (μg) pro kg und Minute	Körpertemperatur (°C)	Zimmertemperatur (°C)
Exp. 3.	13,0 kg ♀	2:40 p.m.					37,9	15
		2:44	Atropin (1 mg pro kg) intravenös injiziert.					
		2:49	1	0,15	0,06	0,009		
		3:22	Carbaminoylcholin (0,4 mg pro kg) intravenös injiziert.					
		0′—1′	2	0,27	2,0	0,54		
		1′—2′	3	0,29	0,06	0,02		
		3:43	4	0,15	0,07	0,01	36,0	14,5
		3:54	Acetylcholin (0,4 mg pro kg) intravenös injiziert.					
		0′—1′	5	0,17	0,35	0,06		
		1′—2′	6	0,09	0,07	0,006		
		4:19	Atropin (1 mg pro kg) intravenös injiziert.					
		4:20	Eserin (0,3 mg pro kg) intravenös injiziert.					
		4:27	7	0,12	0,11	0,01	35,6	14,5
		4:35	Carbaminoylcholin (0,4 mg pro kg) intravenös injiziert.					
		0′—1′	8	0,15	3,7	0,56		
		1′—2′	9	0,06	0,43	0,03		
		4:51	10	0,08	0,13	0,01	35,2	13,5
		5:03	Acetylcholin (0,4 mg pro kg) intravenös injiziert.					
		0′—1′	11	0,17	6,2	1,1		
		1′—2′	12	0,08	0,49	0,04		
Exp. 4.	10,3 kg ♀	2:25 p.m.					37,6	11
		2:30	Atropin (1 mg pro kg) intravenös injiziert.					
		2:37	1	0,19	0,08	0,02		
		2:38	2	0,17	0,10	0,02	37,1	11
		2:52	Carbaminoylcholin (0,4 mg pro kg) intravenös injiziert.					
		0′—1′	3	0,27	2,3	0,62		
		1′—2′	4	0,22	0,08	0,02		
		3:02	5	0,20	0,10	0,02	36,1	11,5
		3:12	Acetylcholin (0,4 mg pro kg) intravenös injiziert.					
		0′—1′	6	0,10	0,37	0,04		
		1′—2′	7	0,13	0,13	0,02		
		3:43					36,3	11
		3:49	Atropin (1 mg pro kg) intravenös injiziert.					
		3:50	Eserin (0,3 mg pro kg) intravenös injiziert.					
		3:57	8	0,28	0,12	0,03	36,5	11
		4:04	Acetylcholin (0,4 mg pro kg) intravenös injiziert.					
		0′—1′	9	0,24	3,6	0,86		
		1′—2′	10	0,27	0,10	0,03		
		4:14	11	0,23	0,10	0,02	36,7	10,5
		4:23	Carbaminoylcholin (0,4 mg pro kg) intravenös injiziert.					
		0′—1′	12	0,31	2,6	0,81		
		1′—2′	13	0,17	0,15	0,03		

* Unmessbar.

Nach einer halben Stunde wurde Eserin gegeben und etwa 8 Minuten darauf wurde Carbaminoylcholin wiederum in einer Dosis von 0,3 mg. pro kg. injiziert. Bald darauf vermehrte sich die Adrenalinsekretion beinahe in demselben Um-

fange wie bei der ersten Carbaminoylcholininjektion; sie betrug 0,20 μg. pro kg. und Minute. Etwa 14 Minuten später wurde Acetylcholin in gleicher Dosis verabreicht. Gleich nach der Injektion wurde eine Steigerung der Adrenalinsekretionsgeschwindigkeit auf 1,1 μg. pro kg. und Minute gefunden.

Den übrigen 3 Hunden wurden 0,4 mg. Carbaminoylcholin sowie Acetylcholin pro kg. intravenös injiziert.

Beim Exp. 2 betrug die vermehrte Sekretionsgeschwindigkeit des Adrenalins, die bald nach der ersten Carbaminoylcholininjektion gemessen wurde, 1,1 μg. pro kg. und Minute. Die Acetylcholininjektion vor der Eserinapplikation vermochte auch bei diesem Versuche keine Vermehrung mehr hervorzurufen. Nach einer halben Stunde wurde Eserin gegeben. Gleich nach der zweiten Acetylcholininjektion, die etwa 15 Minuten nach der Eserindarreichun angestellt wurde, zeigte die Sekretionsgeschwindigkeit des Adrenalins eine hochgradige Vermehrung und erreichte 1,8 μg. pro kg. und Minute. Nach der zweiten Injektion des Carbaminoylcholins wurde die vermehrte Adrenalinsekretion beinahe in demselben Umfange wie bei der ersten Carbaminoylcholininjektion beobachtet; sie betrug 1,2 μg. pro kg. und Minute.

Ähnliche Resultate wurden auch beim Exp. 3 beobachtet. Nach der ersten Carbaminoylcholininjektion vermehrte sich die Sekretionsgeschwindigkeit des Adrenalins bis 0,54 μg. pro kg. und Minute. Die Sekretionsgeschwindigkeit nach der ersten Acetylcholininjektion zeigte nur geringfügige Vermehrung; sie betrug 0,06 μg. pro kg. und Minute. Etwa 25 Minuten später wurde Eserin verabreicht, und dann etwa 15 Minuten darauf wurde Carbaminoylcholin wiederum intravenös injiziert. Dabei zeigte die Blutprobe, die gleich nach der Injektion entnommen wurde, die vermehrte Sekretionsgeschwindigkeit beinahe in demselben Umfange wie bei der ersten Carbaminoylcholininjektion; sie betrug 0,56 μg. pro kg. und Minute. Acetylcholin, das etwa 30 Minuten nach der zweiten Carbaminoylcholininjektion intravenös injiziert wurde, rief sofort eine vermehrte Sekretion des Adrenalins mit dem Wert von 1,1 μg. pro kg. und Minute hervor.

Beim Exp. 4 wurde 0,62 μg. Adrenalin pro kg. und Minute gleich nach der ersten Carbaminoylcholininjektion abgesondert, aber keine Vermehrung der Adrenalinsekretionsgeschwindigkeit war dennoch mehr nach der ersten Acetylcholininjektion nachweisbar. Als Acetylcholin etwa 15 Minuten nach der Eserindarreichung aufs neue injiziert wurde, da ließ sich die Vermehrung der Sekretionsgeschwindigkeit von Adrenalin erkennen; sie betrug 0,86 μg. pro kg. und Minute. Etwa 20 Minuten nach der zweiten Acetylcholininjektion wurde Carbaminoylcholin wiederum eingespritzt. Dabei wurde die vermehrte Sekretionsgeschwindigkeit des Adrenalins in etwas grösserem Umfange als bei der ersten Carbaminoylcholininjektion beobachtet; sie betrug 0,81 μg. pro kg. und Minute.

Zusammenfassung

Bei den mit Evipan-natrium narkotisierten Hunden wurde das Nebennierenvenenblut mit der Lumbalroutemethode entnommen. Die Bestim-

mung der Adrenalinmenge wurde mit Hilfe der kolorimetrischen Methode von Bloor und Bullen ausgeführt. Die intravenösen Injektionen des Carbarminoylcholins und des Acetylcholins in Dosen von 0,3–0,4 mg. pro kg. Körpergewicht wurden vor und nach der Darreichung des Eserins (0,3 mg. pro kg.) angestellt.

Die Verstärkung der adrenalinsekretionsfördernde Wirkung von Acetylcholin durch eine vorangehende Verabreichung von Eserin war in allen Fällen nachweisbar. Dagegen vermochten wir keine unverkennbare Verstärkung der Wirkung von Carbaminoylcholin durch Eserin zu konstatieren.

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The Tohoku Journal of Experimental Medicine, Vol. 68, No. 2, 1958

Refraction of Spreading Induction in the Retina

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(Received for publication, March 28, 1958)

Introduction

In a previous paper by Motokawa and Komatsu[1] it was shown that the velocity of spreading induction depends on the state of adaptation; the velocity was found higher in a retinal area adapted to white light than in the dark-adapted one. In the present experiment colored light was used instead of white light, and it was found that the velocity was decreased below the value in the dark-adapted retina. From these findings it was inferred that the spreading induction would be refracted at the boundary between the dark-adapted and the light-adapted areas of the retina. The present paper deals with various aspects of refraction in the retina.

Experimental

Method

Spreading induction was investigated by our routine method, in which a pair of an inducer and a detector is used. The inducer, I, was a light stimulus for initiating spreading induction. The spreading induction caused by a blue inducer will be called "yellow spreading induction." The detector was a device for detecting arrival of spreading induction at any point at which the detector was placed. It consisted of a patch in hue complementary to the inducer and of a white test patch 1 mm. in diameter. These stimuli were presented always at a distance of 30 cm. in front of the eye of the subject.

The indirect induction established around a yellow detector was measured by the following procedure: The dark-adapted eye was exposed to the yellow patch of the detector for 2 seconds, and then to the white test patch, which was presented at a distance of 1 mm. from the margin of the yellow patch. The interval separating the two photic stimuli has

no influence on the result unless it is not longer than 20 seconds. The electrical excitability of the eye was measured 1.5 seconds after the end of the white test light. The value of electrical excitability was generally higher than that obtained following an isolate presentation of the white test light. The difference between the two values obtained with and without presenting the yellow patch represents the induction effect by the preceding yellow stimulus. The effect was expressed in per cent of the electrical excitability of the dark-adapted retina and designated " contrast effects (C.E.)."

The effect will not be found when the blue inducer was presented at some distance from the detector in the interval of the yellow and the white stimuli (the phenomenon of neutralization). When the spreading induction initiated by the blue inducer is prevented by any way from reaching the detector, the preestablished indirect induction at the detector will remain intact, and in consequence the contrast effect at the detector will be high. Thus the detector serves to detect arrival of spreading induction.

The details of arrangements of patches and experimental procedures will be described together with the results.

Results

Refraction of spreading induction by a light-adapted retinal area prismatic in shape

When the velocity of spreading induction through a prism-shaped retinal image is lower than in the surrounding dark areas, it is to be expected that the spreading induction will be refracted by the image towards the basis of the triangular image. On the contrary, it will be refracted towards the apex of the triangular image when the velocity through the image is higher than in the dark retinal area.

Experimental evidence was provided for the possibilities mentioned above in the following experiment: The arrangement of patches and the sequence of procedures are shown in the inset of Fig. 1. The apical angle of the prism-shaped patch was 15°. The patch was illuminated constantly by yellow light of 10 lux. The beam of spreading induction coming from the blue inducer, I, was refracted by the retinal image of the prism-shaped patch as indicated by a broken line in the inset of Fig. 1. The refracted beam was detected by the detector, D, in the manner described above. The result obtained is shown by a continuous curve marked by solid triangles in Fig. 1. In this case neutralization occurred at a point 17° from the middle point of the detector. The refraction of spreading induction is similar to that of light by a glass prism in this case.

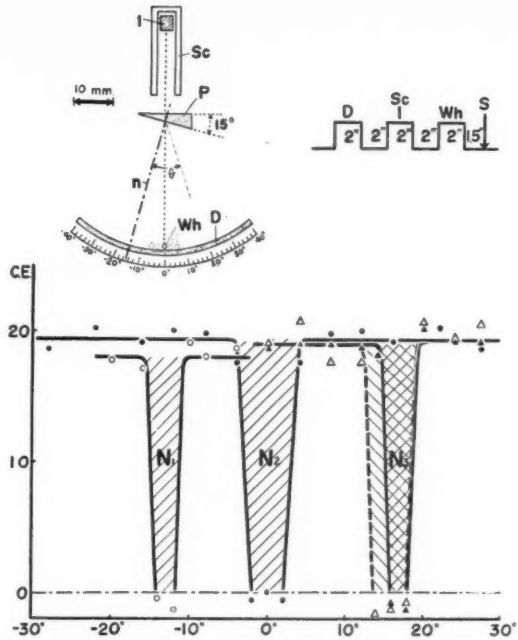

Fig. 1. Arrangement of patches and temporal sequence of experimental procedures for refraction of spreading induction. D : Yellow detector of 150 lux. I : Blue inducer of 150 lux. n : Normal. P : Prism-shaped patch of 10 lux with apical angle of 15°. S : Electric stimulus of 0.1 sec. Sc : White screen of 15 lux. Wh : White test light of 150 lux. Curve N_1 was obtained with P illuminated by white light of 0.07 lux. Curve N_2 represents control obtained without any prism-shaped patch. Curve N_3 was obtained with yellow prism-shaped patch illuminated continuously, and broken curve with the same patch illuminated intermittently.

In some other experiments the illumination was not so continuous as in the experiment mentioned above; the prism-shaped patch, P, was presented together with the yellow detector, D, only for 2 seconds, so that the spreading induction caused by the subsequent inducer, I, traversed the after-image of the prism-shaped patch. The result obtained was qualitatively similar to that obtained in the experiments of constant exposure, as is shown by the broken curve marked by empty triangles in Fig. 1.

Next, white light of 0.07 lux was used instead of yellow light for illumination of the prism-shaped patch. Then refraction occurred towards the apex of the prism-shaped patch, neutralization being found at a point

13° on the opposite side of the middle point of the detector (see the curve connecting open circles).

In the control experiement in which the prism-shaped patch was not presented, the other experimental conditions being the same as above, we found neutralization at the middle point of the detector (see the curve connecting solid circles).

Velocity and refraction of spreading induction as a function of intensities of adapting light

The velocity of spreading induction produced by a blue inducer, I, was measured with the arrangement shown in the inset of Fig. 2. An area, M, which was adapted to yellow adapting light, had to be traversed

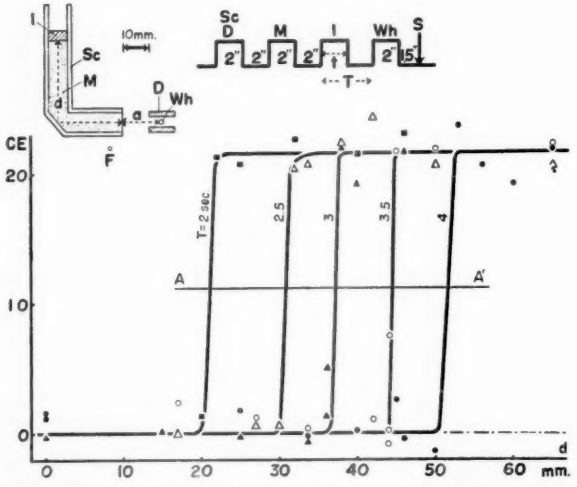

Fig. 2. Measurements of propagation velocity of spreading induction. Labeling of patches the same as in Fig. 1, except for M which served for light adaptation of medium to be traversed by spreading induction. I: Blue inducer. D: Yellow detector. M was illuminated by yellow light of 110 lux.

by the spreading induction initiated by the inducer, I, before reaching the detector, D. While the interval, T, from the onset of I to that of the white test light, Wh, was fixed, the longitudinal distance, d, of M was varied. In this experiment neutralization occurred when the distance, d, was shorter than a certain value, but it failed to occur when the distance was too long. For example, neutralization occurred for d shorter than 20 mm. when T was fixed at 2 seconds. No neutralization occurred for longer distances, because the test of neutralization was made before the spreading induction reached the detector. The critical distance at which

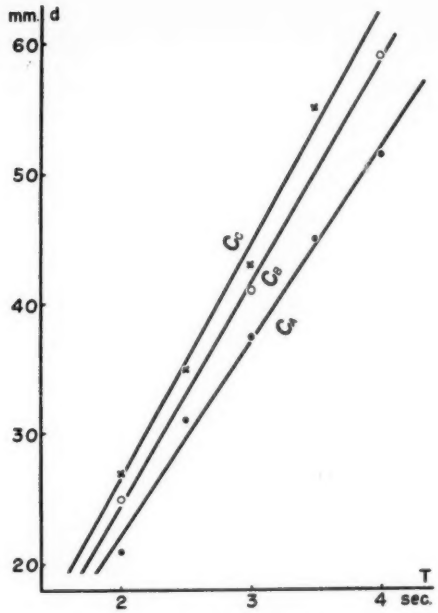

Fig. 3. Relation between conduction distance of spreading induction and conduction time. C_A, C_B and C_C refer to intensities of illumination for M 110, 1.1 and 0.07 lux respectively.

neutralization just failed became greater, as the time interval, T, was lengthened. Such critical distances which are a function of interval, T, will be called " propagation distances." In Fig. 3 propagation distances are plotted against time intervals, T. As can be seen from this figure, the relation is linear within the limits of experimental error, and this means that the velocity was uniform in the light-adapted area, M. The line labeled C_A was obtained with yellow adapting light of 110 lux. Similar experiments were carried out at various adaptation levels of M. The lines C_B and C_C in Fig. 3 refer to adaptation levels at 1.1 lux and 0.07 lux respectively.

The slope of line seems to become steeper as the adaptation level is lowered. In other words, the higher the adaptation level, the slower the propagation velocity.

From the grounds to be stated later, the refractive index can be computed from the propagation velocities in the light-adapted and the dark-adapted retinal areas. On the other hand we can determine the refractive index directly by measurements of refraction in the manner stated

TABLE I

Propagation Velocity of Yellow Spreading Induction in Retinal Area Adapted to Yellow Light

Intensity of yellow light in lux	From experiments of propagation velocity		From experiments of refraction	
	Velocity in retina (mm./sec.) (observed)	Refractive index (calculated)	Velocity (calculated)	Refractive index (observed)
110	0.85	2.00	—	—
10	—	—	0.83	2.02
1.1	0.96	1.77	—	—
0.12	—	—	0.94	1.81
0.07	1.02	1.67	—	—
0.05	—	—	1.08	1.57
0.007	2.00	0.85	—	—

above. Values of refractive index determined by these different methods are compared in Table I. It is to be noted that there is satisfactory agreement between them.

Refraction of spreading induction by a lens-shaped retinal image

A lens-shaped image was formed by a yellow patch of the same form, L, to collect the spreading induction initiated by a blue inducer, I. A white screen, Sc, served to restrict the path of spreading induction to the lens-shaped image (see Fig. 4 A). The detector, D, used was a rectangular blue patch whose direct induction was to be neutralized by the spreading induction from I. A white test patch 2 mm. in diamenter was presented to measure the direct induction caused by the blue patch of the detector. The detector was displaced from experiment to experiment so as to map the distribution of the neutralizing effect of the spreading induction from I. In Fig. 4 A the region at which the spreading induction arrived and neutralized the preestablished direct induction there is marked by open circles, and the surrounding areas in which no neutralization occurred by solid circles. In detail, the open circles represent values of C.E. between -2.0 and $+3.0$, and the solid ones those between 13 and 16. The shaded circles represent intermediate values, that is, those lying between 4.5 and 8.0. It is obvious from this finding that the path of spreading induction is converged by the lens-shaped retinal image in a manner very similar to the action of a convex lens. By a physical convex lens light from a point source is focused on a point where the density of light is higher than at any point on the light path.

The above-mentioned experiment can, however, tell nothing about the density of spreading induction, because neutralization occurs in the

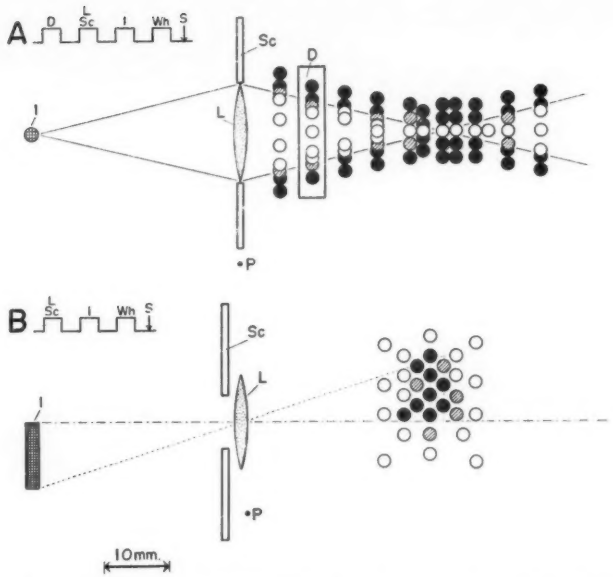

Fig. 4. Convergence of spreading induction by a lens-shaped retinal image. I: Blue inducer. Sc: White screen. L: Yellow lens-shaped patch. D: Blue detector. Upper diagram A represents distribution of neutralizing effect of spreading induction from I upon direct induction caused by D which was placed at various places in field so as to map distribution of neutralization. Occurrence of neutralization is indicated by open circles, and its absence by solid circles. Lower diagram B represents distribution of contrast effects caused by spreading induction collected by L. Contrast effects fully developed are represented by solid circles, and its absence by open circles, shaded ones representing intermediate effects.

manner of all-or-none. If spreading induction is focused on a certain restricted part of the retina an induction effect will become manifest in this part. Based upon this supposition, it was attempted in the following experiment to measure the induction effect which would be caused by spreading induction concentrated by the lens-effect. The procedure used was the same as that employed for measuring the indirect induction around a retinal image: As the inducer was blue in hue the indirect induction caused by it can be measured by the difference of electrical excitabilities at 3 sec. after the sequence of stimuli, blue—white and at 3 sec. after the white stimulus alone. The result obtained is shown in Fig. 4 B. In this figure values of C.E. between -2.0 and $+2.0$ are expressed by open circles. These low values of C.E. indicate that the density of spreading

induction was nearly zero at these points. In a certain restricted region of the retina values of C.E. were as high as from 8.5 to 14.5, as indicated by solid circles. Shaded circles in the figure represent intermediate values of C.E. It is to be noted that the region of high C.E. values just corresponds with the geometrical image of I formed by the lens-action of L. In control experiments in which no lens-shaped patch was presented, values of C.E. were found always zero within the limits of experimental error everywhere on the right side of the screen, Sc.

Refraction of yellow spreading induction by a prism-shaped retinal area adapted to spectral lights of various wave-lengths

In most experiments stated above the prism-shaped patch was illuminated by yellow light, and the refraction of yellow spreading induction by such a yellow prism-shaped image was investigated. In the following experiments spectral lights of various wave-lengths were used for illumination of the prism-shaped patch, the spreading induction being the same as above.

The refractive index was measured after the method of the least deviation angle which is used for determination of the refractive index of a prism in physics. The arrangement of patches and the temporal sequence of procedures are shown in the insets of Fig. 5. The set of patches,

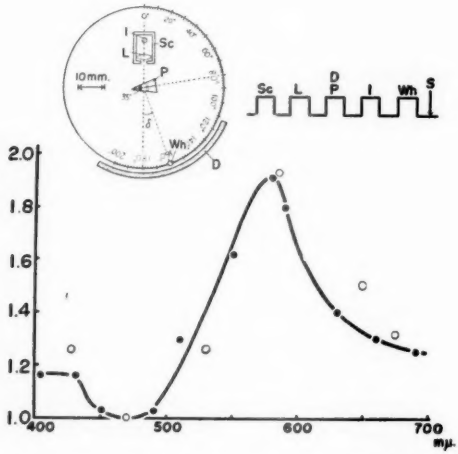

Fig. 5. Measurements of refractive index. Prism-shaped patch, P, was illuminated by spectral lights of various wave-lengths. Set of patches I, Sc and L represents a collimator. Least deviation angle was determined by procedures described in text. Curve marked by solid circles was obtained with refraction by after-image of P, and that marked by open circles with refraction by P during illumination.

I, L and Sc, served to produce a beam of yellow spreading induction. It was refracted by the prism-shaped after-image of patch, P, and reached a certain part of the detector, D. P could be rotated around a point, and the angle of rotation could be read. The prism-shaped patch was placed in such a way that the condition of the least deviation angle was satisfied. The procedures actually used will be explained by an example. In the example to be stated the prism-shaped patch was illuminated with yellow light of 589 mμ. The patch was so placed that the bisecting line of the apical angle pointed to 71° on the scale. The part around $71° \times 2$ of the detector was searched for neutralization. By the method of trial and error we found that for a combination of 74° of the bisecting line and 148° of the detector a complete neutralization occurred. In this case the least deviation angle was given by the difference between 180° and 148°.

If the apical angle and the least deviation angle are denoted by θ and δ respectively, the refractive index, n, will be given by the following formula:

$$n = \frac{\sin \frac{1}{2}(\theta+\delta)}{\sin \frac{\theta}{2}}$$

Putting 35° for θ and 32° for δ in the formula, we obtained 1.82 as the refractive index of the yellow prism-shaped image for the yellow spreading induction.

Similar measurements were carried out with spectral lights of various wave-lengths for illumination of the prism-shaped patch. The results obtained are shown in Table II, and their graphical representation is found in Fig. 5. As can be seen in this figure, the refractive index determined with the yellow spreading induction is highest for a retinal area adapted to yellow light and lowest for an area adapted to blue light.

As the method used above was too complicated, some experiments were performed with the simpler method described in Fig. 1. The illumination of the prism-shaped patch was continuous in these experiments. In spite of these differences in method and condition the results obtained were very similar to those obtained by the complicated method (cf. the curve marked by open circles with that marked by solid ones). It is also to be noted that these two kinds of experiments were carried out by different experimenters on different subjects, and that the dates of the experiments were separated by 6 years.

For comparison some experiments were performed on the propagation velocity, in order to study the dependence of velocity on the wave-length of adapting light for the medium to be traversed by yellow spreading induction. The method used was the same as described in Fig. 2. The

TABLE II

Agreement of Refractive Indices Computed from Propagation Velocities with Those Determined by Experiments of Refraction

Wave-length of adapting light (mμ)	Velocity in retina (mm./sec.)	Refractive index	
		Computed	Observed
690	1.36	1.25	1.24
660	1.30	1.30	1.30
630	1.22	1.40	1.40
589	0.94	1.82	1.81
580	—	—	1.95
550	1.05	1.62	1.62
510	1.30	1.30	1.30
490	—	—	1.03
450	1.67	1.02	1.03
430	1.56	1.09	1.14
405	1.50	1.13	1.14

results obtained are compared with those obtained from the experiments of refraction in Table II.

DISCUSSION

In general, refraction occurs when the conduction velocity in one medium is different from that in another in contact with the former. The refractive index is given by the ratio of the conduction velocities in the two media.

The velocity of spreading induction was determined with three different methods[2),3),4)] with consistent results. It is about 1.7 mm. per second in the dark-adapted retina. As has been shown above, the velocity of yellow spreading induction is lower in the retina adapted to colored light than in the dark-adapted retina. In consequence the refractive index is higher than unity.

On the contrary, the velocity in the retina adapted to white light is higher than that in the dark-adapted retina so that the refractive index is lower than unity. We do not know as yet the reasons why the velocity depends so on the adaptation state of the retina. The mechanism will remain obscure till we get more information about the properties of spreading induction. As has been shown in Tables I and II, we see satisfactory agreement between refractive indices determined by the measurements of refraction and those computed from propagation velocities. This finding together with the facts of reflection of spreading induction[1),4)]

seems to suggest the wave-nature of spreading induction. Experimental evidence will be provided by further experiments on diffraction of spreading induction.

We have seen above that an induction effect occurred in a certain restricted retinal area when spreading induction was focused by the action of a lens-shaped patch. One may ask whether the induction effect is made visible. We also tried to make it visible, but have not yet succeeded in such attempts. The indirect induction around a retinal image is visible as the phenomenon of contrast. In such cases values of C.E. are generally greater than 20 in the region in which simultaneous contrast is observable. With the induction effect caused by the lens-action values of C.E. were 16 at the highest, and such low values of C.E. may not be sufficient to make the effect visible.

Summary

It was shown by the method of electrostimulation of the eye that spreading induction was refracted when a prism-shaped retinal image was formed on the way of its propagation through the retina.

1. The degree of refraction depended on the intensity and also the wave-length of light illuminating a prism-shaped patch whose image was formed on the retina.

2. The propagation velocity of spreading induction was measured to investigate its dependence on the intensity and the wave-length of adapting light. In the retina adapted to white light the velocity was higher than in the dark-adapted retina, while in the retina adapted to colored light the velocity was found lower than in the dark-adapted retina.

3. The refractive index computed from propagation velocities was in satisfactory agreement with that determined by measurements of refraction.

4. It was shown by many examples that the law of geometrical optics holds for spreading induction in the human retina.

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The Tohoku Journal of Experimental Medicine, Vol. 68, No. 2, 1958

Studies on Leucocytosis and Leucopenia in Infectious Diseases

Report V

On Naegeli's Opinion and Significance of Leucocytosis and Leucopenia in Infectious Diseases

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(Received for publication, April 2, 1958)

As discussed in the first—fourth reports[1)-4)], the author has found that the difference between leucocytosis and leucopenia in infectious diseases originates from the diphasis of mitosis of the leucopoietic system in the bone marrow[5-11)].

Concerning the genetic cause of leucopenia many opinion have been voiced. As the cause of leucopenia in typhoid fever, Markoff[12)] reported degeneration of the bone marrow due to toxin of typhoid bacilli, Schur & Löwy[13)] insufficiency of the bone marrow in agreement with Naegeli's[14)] opinion, Jagic & Klima[15)] retardation in the hematopoiesis of the bone marrow and Barta[16)] impediment of new formation of the cells in the bone marrow and disturbance in the outflow of the white blood cells into the peripheral blood.

On the contrary Arneth[17)] regarded as of great importance that the white blood cells were overspent in the periphery. Frank[18)] pointed out some factors which were produced in the spleen and lymphatic apparatuses and disturbed new formation of the cells in the bone marrow.

Baumann[19)] stated lack of some factors which were contained in diet and had the effect of increasing the white blood cells, but in typhoid fever could not be adsorbed well into the body owing to its gastro-intestinal disturbance.

On Naegeli's Opinion

At the present time, however, Naegeli's opinion concerning leucopenia has been widely accepted. He stated that leucocytosis was the result of sufficiency and leucopenia that of insufficiency of the bone marrow.

His opinion is as follows:—leucocytosis is an expression of an activity

of the bone marrow. One speaks of sufficiency of the bone marrow, when the reaction comes out powerfully, and of insufficiency, when it is slight or fails to come out. In the latter the disease can be only mild or unusually severe with serious prognosis. The loosening of this alternative is drawn from the clinical general features. The virulence and possibility of the reaction control leucocytosis, therefore the changes of the white blood cells must take place under some difinite laws, but cannot possibly come out schematically always in the same way because of interplays of various laws. That too explains various findings in the same disease.

As mentioned above the Naegeli's opinion is founded chiefly on the basis of the peripheral blood and postmortem examination of the bone marrow, in exclusion of vital biopsy of the bone marrow. Therefore his opinion ends in vagueness. Against his opinion Schottmüller[20] stated that there were some clinical considerations that must be taken into account and Fujimori[21] reported that in leucopenia there were disturbances in the maturation and outflow of the white blood cells.

The author can't support the Naegeli's opinion on the basis of our previous reports. The considerations that need special discussions are as follows:

1) *The factors of leucocytosis and leucopenia have their respective specificity.* Naegeli did not give notice to the factors causing leucocytosis and leucopenia. For example, there are many clinically transitional states from mild to severe in typhoid, but looked as a whole typhoid is severer than paratyphoid and there are not a few cases of paratyphoid, in which fever appears for a very short time as Fig. 1 shows. Among these paratyphoid group K and A are moderately severe, but B is very mild. This specificity

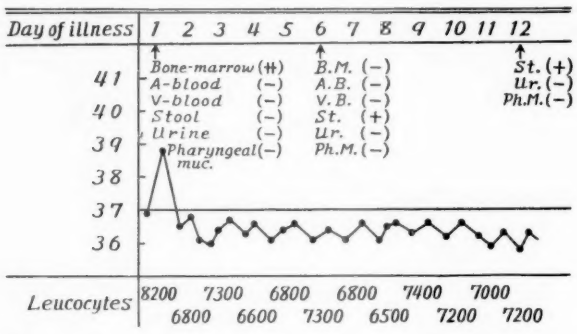


Fig. 1. Mild paratyphoid B case of S.K. (48 years, male). Fever only for six hours on the first day of the illness. Paratyphoid B bacilli were isolated only from the bone marrow after 3 days enrichment in bile media. On the sixth day his fecal culture was positive and he was found to be a fecal carrier.

is seen not only in the clinical courses but also in the biologic nature of their pathogenetic microbes, and in the same way leucopenia is the most remarkable in typhoid fever, followed by paratyphoid K, A and B in the order named. Such a regularly arranged order cannot be explained by Naegeli's opinion, for by his opinion even though leucopenia in severe typhoid can be understood, that in less moderately severe typhoid and paratyphoid cannot be understood.

2) *When factors of different phase have complicated in the course of leucocytosis or leucopenia the white blood cells change their trend toward decreasing or increasing according to the qualitative and quantitative differences of the two phase factors.* When in the course of typhoid fever with an extreme leucopenia an intestinal perforation is complicated and the white blood cells show a trend toward increasing, according to Naegeli's opinion it means that the bone marrow alone turns sufficient from an insufficient state, in spite of serious general conditions and a worst prognosis. This is never to be believed. Furthermore, similar changes in the course of leucopenia in typhoid fever complicated with enterorrhagia, pneumonia, bronchopneumonia and so forth cannot be understood by his opinion.

3) *leucopenia in infectious diseases such as typhoid and paratyphoid group cannot be identified with that in extremely severe cases of pneumonia, perityphlitis, sepsis and others which belong primarily to leucocytosis.* The former is original and primary while the latter is secondary and takes place as a result of insufficiency of the bone marrow. Panmyelophthisis and agranulocytosis are of course results of insufficiency of the bone marrow. Naegeli took primary and secondary leucopenia as a whole for insufficiency of the bone marrow, but in the bone marrow function there is a great difference between primary and secondary leucopenia; it is irritable and reactive in the former, while it is neither irritable nor reactive in the latter. Therefore the former cannot be identified with the latter.

4) *In infectious diseases the changes of the white blood cells in leucocytosis and leucopenia cannot be understood in one and the same phasis.* Naegeli explained the genetic cause of leucocytosis and leucopenia in one and the same phasis, and called an increase of the white blood cells in the blood sufficiency, and their decrease insufficiency of the bone marrow, and he pointed out as the cause of leucopenia insufficiency of the bone marrow due to its exhaustion.

In general, unless complicated in the course of the infectious diseases the white blood cell count shows more than normal values in the first phase, but less than normal values in the second phase, in proportion to the severity of the diseases. This specificity in the change of the white blood cells cannot be explained by Naegeli's opinion.

5) *In the genetic mechanism of leucocytosis and leucopenia Naegeli stated that*

it depended upon the strength of the reaction of the bone marrow, but he did not touch upon its pathogenetic factors. The pathogenetic factors in relation with the bone marrow of the host seem to us to be something like seeds in relation to a farm, i.e., the pathogenetic factors are seeds and the bone marrow is the farm. The quantity of crops depends upon the fertility of the farm but their species (Specificity) depend upon the seeds. therefore the factors of the pathogenetic microbes are very important in the genetic mechanism of leucocytosis and leucopenia in infectious diseases. Heilmeyer[22] states, it seems to him to be probable that the specificity of the toxine of typhoid bacilli results in leucopenia.

As mentioned above, concerning the genetic mechanism of leucocytosis and leucopenia, Naegeli has referred only to the activity of the bone marrow, irrespective of the factors of the pathogenetic microbes. In infectious diseases these factors are highly specific concerning changes of temperature, respiration, pulsation and eruption of rash, appearance of brain symptoms and other biologic reactions of the human body. Therefore a sure or probable diagnosis can be frequently established on the basis of these several symptoms, particularly in the clinic the change of the white blood cells are regarded as of great importance in making diagnosis, for their change are specific to the respective diseases.

Faludi[23] has taken leucopenia for suppuration of the blood, and Rohr[24] has stated his opinion in his work as follows: according to Faludi's new researches it is probable that leucopenia can take place partly by destruction of the white blood cells in the blood vessels even in spite of accelerated new formation, but on the other hand leucopenia can be a secondary sequence of an injury to the bone marrow, to which fact Naegeli has already called attention in 1900 on the basis of his classic studies on the blood picture of typhoid fever. The difficulty in classifying the findings of the bone marrows in leucocytosis and leucopenia is based upon the fact that the systematic examinations in the course of infectious diseases are relatively few, particularly few in human subjects.

In my examinations it was found that there is not much difference in the destruction of the white blood cells both in leucocytosis and leucopenia.

Significance of Leucocytosis and Leucopenia Observed from the Standpoint of the Hematopoiesis in the Bone Marrow

In the early stage of infectious diseases both in leucocytosis and leucopenia a symptomatic leucocytosis with a nuclear shift to the left is seen in the blood. On the other hand the bone marrow is irritated by the stimulus of the pathogenetic microbes and reacts physiologically against them with

a rise or drop of mitosis in the bone marrow, according to the specificity, quality and quantity of their factors. Consequently the nucleated cells in the bone marrow increase in cases with the first-phase factors and decrease in those with the second-phase factors.

The increase and decrease of the nucleated cells in the bone marrow appears in the peripheral blood with a nuclear shift to the left, showing leucocytosis in the former and leucopenia in the latter. Thus the blood picture is a reflected image of the bone marrow picture.

Both in leucocytosis and leucopenia, there is not much difference in the maturation, the outflow into the peripheral blood and the destruction of the white blood cells. It goes without saying that in infectious diseases some disturbance in the maturation of the nucleated cells is seen in the bone marrow, and the destruction of the white blood cells is accelerated in the couse of the illness, particularly in the peak stage. But these findings can be observed equally both in leucocytosis and leucopenia, according to the severity of the diseases, and it can not be found that the destruction of the white blood cells in the peripheral blood is accelerated more vehemently in the latter than in the former.

The most remarkable difference between leucocytosis and leucopenia is observed in their mitotic function in the bone marrow; in leucocytosis it is frankly accelerated, while in leucopenia it is equally frankly lowered. As the disease improves, the function gradually becomes lowered in the former but accelerated in the latter, attaining the normal level in the reconvalescence. This difference originates from the diphasis of mitosis of the leucopoietic system in the bone marrow.

In infectious diseases the significance of leucocytosis can be easily understood on the basis of the function of leucocytes in the defense mechanism of the body, but leucopenia seems to us to be inconsistent with their function, for the decrease of leucocytes means a drop of the defense force against pathogenetic agents. This question must be considered from the standpoint of bacterio-immunology instead of hematology.

In the change of leucocytes there are obvious differences between that in coccal and in bacillary infection; the increase of leucocytes is more remarkable in the former than in the latter, but production of the immune body is lower in the former than in the latter. Fig. 2 indicates the relation between the changes of white blood cell counts and titers of agglutination, obtained through 1727 examinations in the course of 536 typhoid cases. At the onset of the illness, the white blood cells in the periphery increased as a symptomatic leucocytosis and then they decreased gradually in the course of the illness, showing its minimal value (mean value) of 4400 in the fever-abating stage (the 4th week) of the illness. On the other hand, in the beginning of the illness the Widal's reaction presented nothing

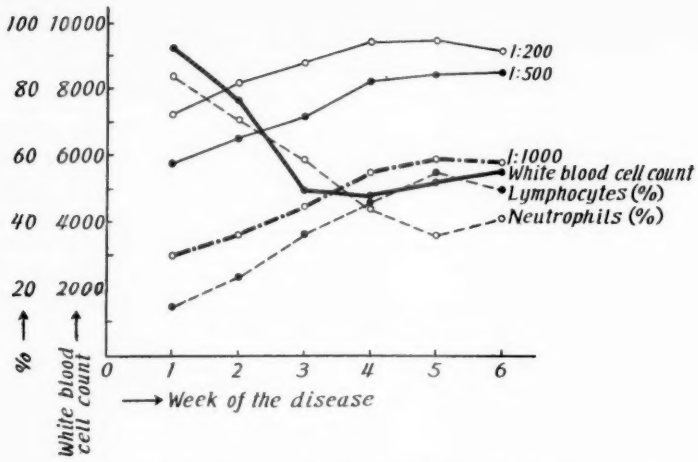


Fig. 2. Relation between the changes of white blood cell counts and titers of agglutination of the peripheral blood, obtained through 1727 examinations in the course of 536 typhoid cases.

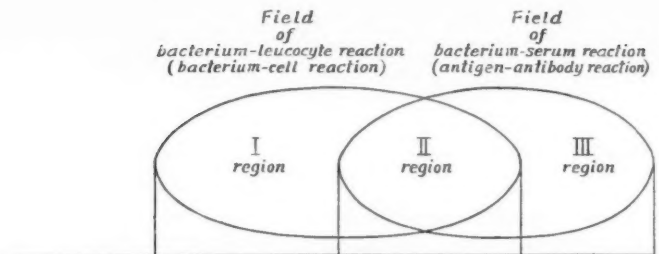
characteristics, but became positive during the second week and reached its maximal height at about the 4th to 6th week, and then decreased rather gradually. Therefore it shows strikingly that the white blood cell counts inversely proportionate to the titers of agglutination.

It is a noteworthy fact that the production of immune body is, in general, conspicuous in the case of leucopenia of infectious diseases.

In the defense mechanism of the body the defense force of the blood consists of 2 forces: the one is the defense force of the white blood cells and the other of serum. The former force is inversely proportionate to the latter, and sum of these 2 forces, i.e., the total of the defense forces of the blood is much the same both in leucocytosis and leucopenia.

Accordingly there are 2 fields of the defense reaction of the blood: the one is the field of cell-bacterium reaction and the other of antibody-antigen reaction. Against the pathogenetic agents the leucocytes work in the former, while the antibodies in the serum react in the latter, as Fig. 3 shows.

Which of these 2 fields is chosen as the chief place of the defense reaction of the blood depends upon the specificity of the pathogenetic microbes. If the defense reaction takes place chiefly in the field of leucocyte-bacterium reaction (cell-bacterium reaction), so a marked leucocytosis appears and on the contrary, if the reaction occurs chiefly in the field of serum-bacterium reaction (antibody-antigen reaction) a re-



Diseases		Infectious diseases and purulent diseases due to cocci, rickettsial and viral diseases	A group of bacillary diseases, various kinds of tuberculoses, viral diseases	Bacillary, rickettsial and viral diseases
Defense force of the blood	Defense force of blood cells	+++	++	+
	Defense force of serum	+	++	+++
Leucocyte reaction		Leucocytosis	normal	Leucopenia

Fig. 3. Defense reaction of the blood.

markable leucopenia comes out.

On the region of the overlapping fields (Fig. 3, 11 region), the leucocyte counts show about normal values. This is the reason why the leucocyte counts in various diseases due to tuberculous bacilli show about normal values.

Furthermore in infectious diseases the defense reaction of the blood in the case of leucopenia does not react in the third region (Fig. 3, 111 region) from the very onset of the illness. For example, the defense reaction of the blood in leucopenia of typhoid is as follows:—at the onset of the illness before immune bodies are produced, leucocyte-bacterium reaction takes place on the first region (Fig. 3, 1 region). Accordingly in response to the peripheral demand the reserved leucocytes flow actively out of the bone marrow into the peripheral blood, showing a symptomatic leucocytosis in the case of leucopenia as well as of general leucocytosis. In the later course of the illness the production of the immune bodies is accelerated in the reticulo-endothelial system of the entire body, but on the contrary the leucocytes decrease gradually. Consequently for a while the defense reaction takes place on the second region (Fig. 3, 11 region).

After that the production of the immune bodies is accelerated more and more, and in the 3rd to 6th week of the illness the titer of agglutination (Widal's reaction) indicates the largest presence of the immune bodies in

serum of the patient; while, on the contrary, leucocytes decrease to the minimal values in the 3rd to 4th week of the illness. Thus the defense reaction takes place on the third region (Fig. 3, 111 region).

As above-mentioned, the titer of the immune bodies is inversely proportionate to the leucocyte count.

Therefore, observed from the angle of the white blood cells, leucocytosis is nothing but a picture of cell-bacterium reaction and leucopenia that of antibody-antigen reaction.

It is clear that leucopenia in infectious diseases signifies neither an insufficiency of the bone marrow nor a drop of the defense force of the body.

In the defense mechanism of the body the diphasis of mitosis of the leucopoietic system is absolutely necessary and indispensable to the bone marrow to comply with both the cell-bacterium reaction and the antibody-antigen reaction. That is to say, it is related to the diphasis of mitosis of the leucopoietic system that there are 2 fields of the defense reaction of the blood in the body.

On the other hand, the function of the erythrocytes is exchange of gases, i.e., absorption of oxygen an release of carbon dioxide, and except them no one can do this metabolism so the erythrocytes try as far as possible to keep themselves always in their normal count, and when anemia has occured, the mitosis of the erythropoietic system in the bone marrow is accelerated at once. Therefore, it is a matter of course that the mitosis of the erythropoietic system in the bone marrow is monophasic, for erythrocytes have only one field for their function.

It is a misconception to take leucopenia for insufficiency of the bone marrow, for in infectious diseases, unless functionally or organically injured severely, the bone marrow is excited sensitively and reacts promptly, according to the stimulus of the pathogenetic microbes in leucopenia as readily as in leucocytosis. These changes of the bone marrow function are parallel to the clinical symptoms. Therefore it may be said, leucocytosis and leucopenia are both consequences of excited function of the bone marrow, as Fig. 4 shows.

It shows a favourable course of the disease with a good prognosis, unless too extreme, to see an increase of the white blood cells in the course of a disease, which usually accompanies leucocytosis and their decrease in the course of a disease, usually accompanying leucopenia. On the contrary it is not a favourable course of the disease, unless it is very mild, to find the white blood cells decrease in the course of the disease, which as usual shows leucocytosis, because it proves insufficiency of the bone marrow or complications with a serious prognosis and similarly not favourable, to find them increase in the course of the disease, which as usual shows

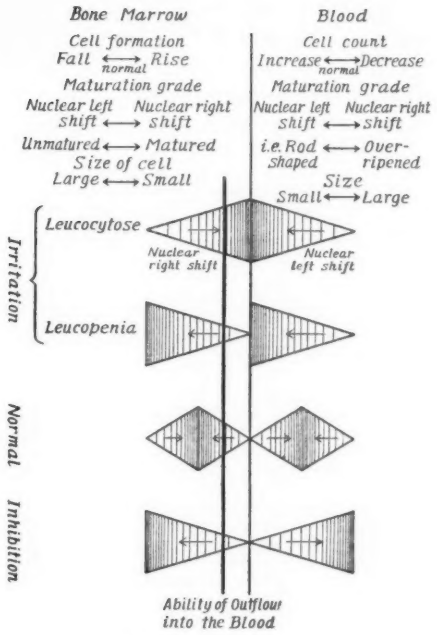


Fig. 4. Schema of cell formation and mechanism of its regulation.

leucopenia, because it argues complications.

If the white blood cells do not increase in the course of severe typhoid fever case with an extreme lecucopenia notwithstanding complication by perforative peritonitis, severe pneumonia etc., it signifies insufficiency of the bone marrow with a serious prognosis. These cases are similar to those of severe pneumonia, perityphlitis, sepsis and others, in which the white blood cells decrease as a result of insufficiency of the bone marrow.

Here it is noteworthy that the hematopoietic function of the bone marrow varies considerably according to individual dispositions.

Table I shows the changes both in the bone marrow and the peripheral blood in every stage of leucocytosis and leucopenia in infectious diseases.

The bone marrow picture of normal person is shown in Table II and the relation between mitosis index in the bone marrow and the leuco cyte count in the peripheral blood is presented in Table III.

By simply examining the leucocyte count and the blood picture of

the periphery in collation with Table I–III, one can guess the change and the picture of the bone marrow in infectious diseases without taking bone marrow, which gives patients uneasiness and pain, because the peripheral blood is the reflected image of the bone marrow.

My opinion on the hematopoiesis of the bone marrow in leucocytosis

TABLE I

Bone Marrow and Blood Pictures in Every Stage of Infectious Diseases

	Fever stage	1 phase	2 phase	Similarity
Bone marrow	Onset	Mobilization of the reserved cells into the peripheral blood. A symptomatic nuclear shift to the left	The same as 1 phase	Mobilization of the reserved cells. A symtomatic nuclear left shift
	Fever-rising	A rise of M. with an increase of the cells takes place in the primitive cells and gradually in the more mature ones. A nuclear shift to the left gradually to the right. An increase of the nucleated cells	A drop of M. with a decrease of the cells takes place from the left and later gradually to the right. A remarkable nuclear shift to the left. A decrease of the nucleated cells	Specificity of the two phases becomes remarkable
	Acme	M. and M.I. rising highest, the center of mitosis shifts most to the right. The largest increase of cells on the right. The most remarkable increase of the nucleated cells. A most apparent relative lymphopenia. The nuclear shift to the right most remarkable. Degeneration and destruction of the cells most evident	M. and M.I. drop lowest. The drop of mitosis shifts most to the right. The largest decrease of cells on the right. The most remarkable decrease of the nucleated cells. A most apparent relative lymphocytosis. The nuclear shift to the left most remarkable. Degeneration and destruction of the cells most evident	Specificity of the two phases most striking. Degeneration and destruction picture most remarkable
	Fever-abating	A drop of M. and M.I., the center of M. shifted from the right to the left. A decrease of the nucleated cells. An increase of the eosinophil and basophil cells. A nuclear shift to the right and then gradually to the left	A rise of M. and M.I.. An increase of the cells from the left to the right. An increase of the nucleated cells. An increase of the eosinophil and basophil cells. The nuclear shift to the left reduced	Destruction and degeneration diminished. An increase of the eosinophil and basophil cells
	Reconvalescence	M. drops to normal. M. centers first passing over to the half-matured, then to matured myelocytes and finally over to the half-matured myelocytes as in normal bone marrow. The nucleated cells decreases to normal	M. rises to normal. A rise of M. appears in the more matured myelocytes. An increase of the nucleated cells nearly to normal value	Return to normal

(*Concluded next page*)

	Fever stage	1 phase	2 phase	Similarity
Peripheral blood	Onset	Symptomatic leucocytosis with a nuclear left shift	The same as 1 phase	Symptomatic leucocytosis with a nuclear left shift
	Fever-rising	An increase of the leucocytes with a nuclear left shift	A decrease of the leucocytes with a nuclear left shift	A nuclear shift to the left
	Acme	The most remarkable leucocytosis with a nuclear left shift. Relative lymphopenia. Degeneration and destruction remarkable	The most remarkable leucopenia with a nuclear left shift. Relative lymphocytosis. Degeneration and destruction remarkable	A nuclear left shift. Degeneration and destruction remarkable
	Fever-abating	Leucocytosis alleviated. A nuclear shift to the right. Eosinophil and basophil leucocytes appear	Leucopenia alleviated. A nuclear shift gradually reduced. Eosinophil and basophil leucocytes appear	Eosinophil and basophil leucocytes appear
	Recon-valescence	Return to normal. Post-infectious eosinophilia and basophilia. Appearance of over-ripened neutrophil and eosinophil leucocytes	A nuclear left shift gradually reduced and returned to normal. Post-infectious eosinophilia and basophilia	Return to normal. Post-infectious eosinophilia and basophilia

TABLE II

Bone Marrow Picture of Normal Cases

Systems	Cells			%	%	%
Reticulo-endothelial	Macrophages			0–0.1	7.1	7
	Plasma cells			2.5		
	Lymphoid cells			4.5		
Erythropoiesis	Basophil			3.0	27.4	27
	Polychromatic			20.0		
	Oxyphil			4.4		
Leuco- and lymphopoiesis	Neutrophils	Myeloblasts		2.0	2.0	100
		Myelocytes	Unmatured	2.6	77.4	
			Half-matured	6.4		
			Matured	10.2		
		Metamyelocytes		12.2		
		Rod-shaped		32.1		
		Segmented		12.9		
	Eosinophils			4.7	20.7	
	Basophils			0.2		
	Monocytes			2.8		
	Lymphocytes			14.0		

TABLE III

The Relation Between the Mitosis Index and White Blood Cell Count in the Peripheral Blood

White blood cells		2001-6000	6001-9000	9001-13000
		Leucopenia	Normal	Leucocytosis
M.I.	‰	1.90-2.38	2.20-3.10	2.60-4.30
Mean value	‰	2.17	2.50	3.20

TABLE IV

Hematopoiesis of the Leucopoietic System of the Bone Marrow in Infectious Diseases

- Hematopoiesis of the bone marrow
  - 1. Sufficiency
    - 1. Rise of mitosis . . . The first phasis . . . Leucocytosis
    - 2. Drop of mitosis . . . The second phasis . . . Leucopenia
  - 2. Insufficiency
    - Organic
    - functional . . .
      - 1. Extremely severe cases, which primarily belong to leucocytosis but show remarkable leucopenia: sepsis, pneumonia, perityphlitis.
      - 2. Extremely severe cases of leucopenia, in which the white blood cells do not increase not-with-standing severe complication by perforative peritonitis or pneumonia.

and leucopenia in infectious diseases is shown in Table IV.

CONCLUSION

At the present time, Naegeli's opinion concerning the genetic mechanism of leucocytosis and leucopenia in infectious diseases has been widely adopted. The author has harboured doubts about Naegeli's opinion and during these eighteen years 1940–1958 made studies on the genetic cause of leucocytosis and leucopenia.

In our systematic serial examination of the hematopoietic function of the bone marrow in the course of various infectious diseases, the author has found that the difference between leucocytosis and leucopenia originates from the diphasis of mitosis of the leucopoietic system in the bone marrow.

In infectious diseases, unless it is functionally or organically injured severely, the bone marrow is stimulated sensitively and reacts promptly according to the specificity of the first- or second-phase factors of the pathogenetic microbes, showing a rise or drop of mitosis. An increase or decrease of the nucleated cells in the bone marrow is reflected in the peripheral blood, showing leucocytosis and leucopenia.

Here it is very important to note the fact that, besides pathogenetic microbes, there are many physico-chemical agents which stimulate the

bone marrow. When the bone marrow is stimulated by these agents, the red blood cells are formed in the monophasis of mitosis of the erythropoietic system and the white blood cells in the diphasis of mitosis of the leucopoietic system, as long as the bone marrow is not insufficient.

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The Tohoku Journal of Experimental Medicine, Vol. 68, No. 2, 1958

Biochemical Studies on Carbohydrates

CCXVI. On Conditions of Acetolyzing Molisch-positive Mucopolysaccharides

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(Received for publication, April 3, 1958)

Since Franchimont[1] applied acetolysis to cellulose in 1879, the conditions for fission in that way of cellulose[2] but not of Molisch-positive mucopolysaccharides have been investigated scrupulously. In the structural study of the group mucopolysaccharide from pig stomach mucus,[3,4] we applied the procedure, which Bergmann *et al.*[5] had used for acetolysis of chitin, and its minor modifications. However, oligosaccharide acetates were thereby yielded little as compared with monosaccharide acetates, and besides, a large amount of the material remained unattacked. Hence we have investigated how to raise the yield of oligosaccharides particularly disaccharides by the experimental scheme following: 1) acetylation of a Molisch-positive mucopolysaccharide in order to change it soluble in the acetolysis reagent, 2) standing portions of the mucopolysaccharide acetate with a glacial acetic acid-acetic anhydride mixture (1:1 by volume) containing a various amount of H_2SO_4 at different temperatures for different durations of time, 3) deacetylation of the products, and 4) column chromatography of the deacetylation products to compare the yields of various fractions monosaccharides, disaccharides, oligosacchrides excepting disaccharides, and polysaccharides with one another. The results indicated that the best yield of disaccharides is obtained when acetolysis is effected with the glacial acetic acid-acetic anhydride mixture containing 4% H_2SO_4 at 30°C for 4 days and that of oligosaccharides exclusive of disaccharides is given when the H_2SO_4 concentration is increased to 8% and acetolysis is continued for 15 days at 20°C. Under the latter conditions the disaccharide fraction is produced in quite good yield simultaneously.

Experimental

Material and Method

1) Material. The group mucopolysaccharide from pig stomach

mucus which had been prepared according to Sinohara,[4] was used as the material.

2) Method. *a*) *Preliminary acetylation of the mucopolysaccharide.* The procedure of Carson and Macley[6] was adapted as follows. 10 g. of the mucopolysaccharide was taken up in 50 cc. of formamide and shaken at room temperature for 30 minutes, whereby it dissolved clearly. To the solution, 100 cc. of dry pyridine was added slowly at room temperature at first and afterwards 90 cc. of acetic anhydride was added in small portions during 20 minutes under cooling on ice. The mixture was shaken at room temperature for 1 hour and then stood at 30°C for 2 days with occasional shaking. The reaction mixture tinged yellowish was poured into 3 *l.* of 2% HCl containing about 500 g. of smashed ice, stirred for 1 hour and centrifuged. The centrifugate was washed with 100 cc. of ice-chilled 0.5% HCl and two 100 cc. portions of ice-chilled water, suspended in 100 cc. of water and placed in a refrigerator. Centrifuged. The centrifugate here was dried at 35°C under reduced pressure over solid caustic soda and anhydrous calcium chloride. 12.1 g. of a white powder was obtained. It had $[\alpha]_D^{20}$ of $\frac{-0.503\times 100}{1.65\times 1}=-30.5°$ in a mixture of methanol and chloroform in the volume proportion of 1 : 1. N (micro Kjeldahl) 3.5%, acetyl (Suzuki[7]) 33.0%, hexosamine (Masamune and Yosizawa[8]) 28.1%, galactose (Reaction HR_{10} of Masamune and Sakamoto[9]) 19.9%, L-fucose (a modification of Dische and Shettles[10]) 9.8%, sialic acid as $C_{11}H_{19}NO_{10}$ (Werner and Odin's direct Ehrlich reaction[11]) 0.9%, ash (Pregl method without use of H_2SO_4) 0%.

b) *Acetolysis of the acetylated mucopolysaccharide.* A typical example will be described. 9 g. of the mucopolysaccharide acetate was taken up in 90 cc. of a mixture of equal volumes of glacial acetic acid and acetic anhydride and shaken at room temperature for 30 minutes until it dissolved transparently. The solution was chilled on a freezing mixture (about −5°C), 4 cc. of concentrated sulfuric acid was added drop by drop under agitation, and the mixture was stood at room temperature for 2 hours. Turbidity that had occurred on the addition of sulfuric acid disappeared. The solution was further stood in a thermostat at 30°C with occasional shaking. When 4 days had elapsed, a 30 cc. portion of the solution was poured into a mixture of 1 *l.* of 60% (v/v) methanol containing 150 g. of smashed ice and 300 g. of Amberlite IR 45 (OH form) under vigorous agitation, agitated continuously for 30 minutes and further stood at room temperature for 6 hours. Filtered. The resin on the funnel was washed with three 100 cc. portions of methanol. The filtrate and washings were united and distilled in vacuo to about 100 cc. To the condensate was added 100 cc. of methanol, with following distillation to

100 cc. under reduced pressure. This operation was repeated several times to expel the acetic acid, and then the solution was distilled to dryness and placed in a desiccator. 3 g. of a yellowish brown powder was given. From the balance of the reaction mixture above, 30 cc. portions were taken out at 4 days' intervals to treat similar to above. The yields of the acetolysis products here were also 3 g.

c) *Deacetylation.* It was conducted according to Kuhn and Kirschenlohr.[12] 3 g. of an acetolysis product was dissolved in 60 cc. of absolute methanol, 60 cc. of methanol saturated with NH_3 at 0°C was added, and the mixture was placed in a thermostat at 20°C for 6 hours with subsequent distillation in vacuo at below 20°C. The still-residue was dissolved in 20 cc. of methanol and again distilled. The residue thus given was taken up in 15 cc. of water, the insoluble part was rejected, and the solution was added to 1.5×3 cm. columns of Dowex 50 (H form) and of Amberlite IR 45 (OH form) in turn and washed with 50 cc. of water. The effluent and washing were distilled in vacuo to dryness and placed in a desiccator. About 1.3 g. of a yellowish brown powder was yielded from each of the acetolysis products.

d) *Column chromatography of the deacetylated products.* The procedure of Whistler and Durso[13] was adapted, employing a column packed of charcoal alone. Namely, 1.3 g. of a deacetylated acetolysis product was dissolved in 10 cc. of water, added to a 3×10 cm. column and eluted by the aid of two 1 *l.* portions of water, 1.5 *l.* each of 2.5% (v/v), 5% and 7.5% ethanol and 2 *l.* each of 10%, 20%, 30% and 50% ethanol and 50% acetone in succession. The consecutive effluents were separately distilled under reduced pressure to dryness. All the still-residues were subjected to determination of iodine use (See *e*)), and those of the first two eluates to paper chromatography of sugar components in addition.

e) *Determination of iodine use.* The method of Whistler and Tu[14] was modified as follows. 20–30 mg. substance was dissolved in 1 cc. of water and bufferized with 3 cc. of the Na_2HPO_4-NaOH buffer of pH 11.2 and I 0.54, and immediately thereupon 2 cc. of 0.05 *N* I_2 was added. The mixture was hermetically stoppered, stood in a water-bath at 20°C for exactly 30 minutes avoiding light, acidified with 1 cc. of 1 *N* H_2SO_4 and titrated with 0.01 *N* $Na_2S_2O_3$. The control run with glucose and the blank were carried out in parallel.

Results with Comment

1) The still-residue of the first effluent in *d*) of the second section showed spots of L-fucose, N-acetylhexosamine and galactose in a paper chromatogram developed by descending irrigation with Solvent butanol-

TABLE I

Yields and Analyses of Various Fractions of the Deacetylated Products, when the Mucopolysaccharide Acetate Was Acetolyzed under Various Conditions

Combined conditions of acetolysis																		Analysis	
No.			I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	
Temperature (°C)			20					30								40			
Concn. of H_2SO_4 (w/v)			2	4	4	8	8	2	4	4	8	8	8	12	16	4	4	8	
Duration (days)			15	15	30	15	30	4	4	12	4	8	12	8	8	4	12	4	
Fr.	Solvent		Yield in per cent of total sum																
	Name	Volume (*l.*)																	
A	Water	1.0	8.0	22.2	27.2	13.0	27.0	9.5	23.5	33.2	25.8	30.5	38.2	54.0	65.0	33.4	42.0	37.2	91.0	0.5
B	Water	1.0	3.0	3.0	4.3	5.0	5.8	2·8	4.2	2.2	6.0	6.1	4.8	3.0	3.0	4.0	3.7	5.5	54.5	0.0
	2.5% (v/v) ethanol	1.5	4.9	4.5	8.8	9.3	7.4	4.0	8.1	10.5	10.0	10.0	10.1	6.2	5.0	11.3	9.3	8.2	45.6	0.0
	5% (v/v) ethanol	1.5	3.8	5.2	4.5	8.8	5.9	4.2	10.1	6.4	5.4	4.0	3.2	4.3	4.1	6.6	5.9	6.5	41.9	0.0
	7.5% (v/v) ethanol	1.5	3.4	5.1	5.0	6.9	5.7	4.8	9.3	4.6	5.9	5.2	4.2	4.2	4.1	6.4	4.7	6.3	40.8	0.0
C	10% (v/v) ethanol	2.0	5.5	5.6	2.4	7.8	8.9	4.8	7.5	3.7	5.2	5.3	5.6	4.8	2.9	5.9	5.2	5.9	36.0	0.0
	20% (v/v) ethanol	2.0	5.2	14.2	12.8	7.4	10.3	8.1	7.0	12.4	10.8	8.3	6.9	3.7	3.0	5.9	7.5	7.1	31.9	0.0
	30% (v/v) ethanol	2.0	4.5	4.5	2.5	3.0	5.8	7.0	3.5	4.9	4.6	3.1	3.3	2.6	2.3	4.0	6.6	4.2	21.4	0.0
	50% (v/v) ethanol	2.0	6.8	6.6	9.5	6.8	5.5	10.2	3.5	1.2	1.2	1.0	2.2	3.7	2.7	3.2	2.2	3.2	16.0	0.0
	50% (v/v) acetone	2.0	7.8	7.4	6.2	8.0	5.2	5.5	1.7	4.3	1.1	2.8	1.9	1.6	3.0	1.5	1.6	4.6	15.3	0.0
D			47.1	27.1	16.8	24.0	12.5	39.1	21.6	17.6	24.0	23.7	19.6	11.9	4.9	18.8	11.3	11.3		

* Pregl method without use of H_2SO_4.

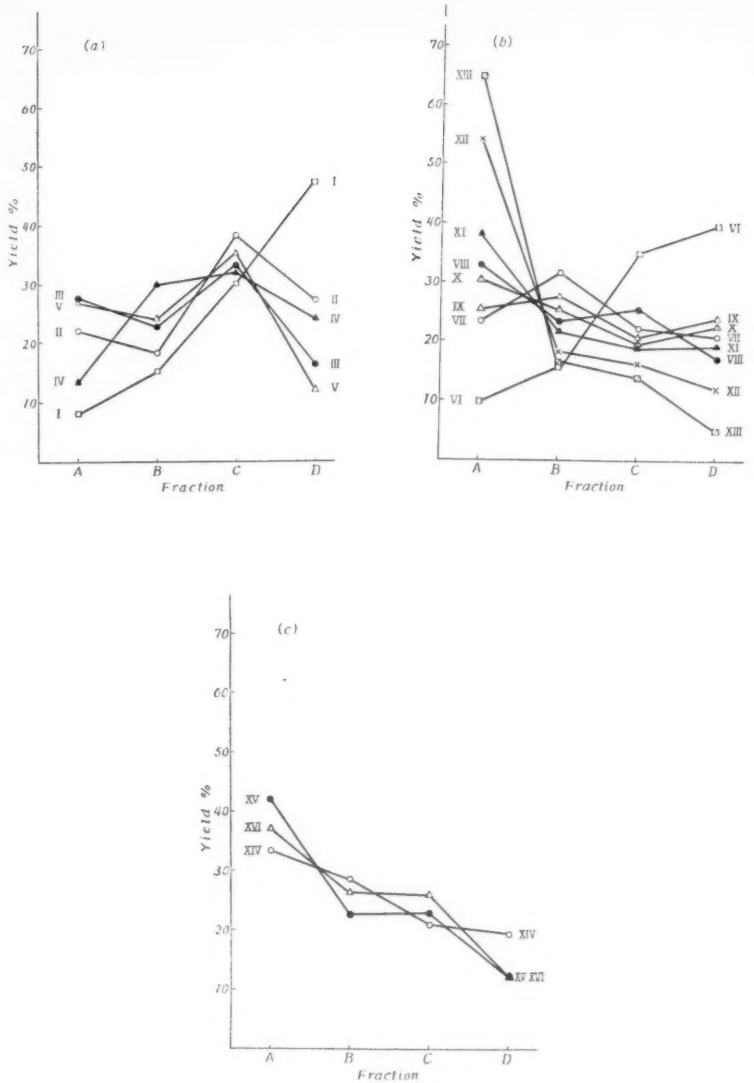

Fig. 1. Yields of Fr. A, B, C and D in per cent of the respective deacetylated products when the mucopolysaccharide acetate was acetolyzed under combined conditions Nos. I—V (temp. 20°C) (*a*), Nos. VI—XIII (temp. 30°C) (*b*) and Nos. XIV—XVI (temp. 40°C) (*c*).

* Pregl method without use of H_2SO_4.

pyridine-water (5:3:2)[15] (Toyo Roshi filter paper No. 3; 25°C; spray reagent the aniline hydrogen phthalate reagent of Partridge), and that of the second effluent gave a spot with $R_{lactose}$ of 1.33 in a paper chromatogram developed in a similar manner.

2) Yields of the fractions are embodied together with their iodine uses in Table I and graphically expressed in Figs. 1a—1c. The fractions reducing iodine as much as 90, 55–40 or 40–15% as glucose and that which was not eluted with 50% acetone are designated Fr. A, B, C and D respectively. The amount of the last fraction was calculated by subtracting the sum of Fr. A, B and C from the amount taken for the chromatographic fractionation.

Acetolysis at 20°C. 2% H_2SO_4 is too weak for sufficient acetolysis. Fr. C predominated in yield when 4% and 8% H_2SO_4 were employed.

The use of 8% H_2SO_4 at 20°C for 15 days is most suited to obtain Fr. C. Thereby Fr. B is yielded in a quite large amount though not so large as is given under combination number VII of conditions (See Table I), so that those conditions are recommendable for separating Fr. B as well as Fr. C at the same time.

Acetolysis at 30°C. The yields of Fr. A and B were small, when 2% H_2SO_4 was used. On the contrary, a great part of the material was broken up into monosaccharides by 12% or 16% H_2SO_4. 4–8% are appropriate concentrations of H_2SO_4 to obtain Fr. B. In this case, the yield of Fr. A is raised a little by prolongation of the duration of acetolysis at the expense of Fr. B but those of Fr. C and D are not.

Fr. B is yielded most abundantly, when acetolysis is effected with 4% H_2SO_4 at 30°C for 4 days.

Acetolysis at 40°C. Most of the material was disintegrated into Fr. A by 4 and 8% H_2SO_4, the other fractions particularly Fr. D being decreased. 40°C is therefore not adequate for effective acetolysis to obtain Fr. B or C.

Duration of acetolysis. It has no marked influence upon the relative amounts of the fractions given, suggesting that equilibrium takes place when acetolysis has proceeded to a certain extent.

Summary

The group mucopolysaccharide from pig stomach mucus was acetolyzed with reagents containing various amounts of H_2SO_4 for various durations at various temperatures to determine the best conditions for obtaining disaccahrides and oligosaccharides excepting disaccharides.

My thanks are due to the Ministry of Education for a grant given through the Grant Committee for Scientific Researches. H. Masamune.

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The Tohoku Journal of Experimental Medicine, Vol. 68, No. 2, 1958

Molisch-positive Mucopolysaccharides of Gastric Cancers as Compared with the Corresponding Components of Gastric Mucosae

Third Report

On MPS (Mucopolysaccharide) II*

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This account deals with " MPSs II " of gastric cancer and of gastric mucosa which have been prepared as described in the Frist Report.[1] They closely resemble each other regarding biological and physical properties. With respect to the chemical findings, however, it was noticed that glucosamine/galactosamine ratio and contents of glucose and of sialic acid are higher in the cancerous MPS II than in the non-cancerous, while the L-fucose contents of the two mucopolysaccharides are in reversed relationship. The other sugar components show almost no quantitative discrepancy between the two substances.

Experimental

Electrophoretic examination. As solvents were employed 0.1 *M* veronal-HCl buffer, pH 8.6, I 0.1 +NaCl (0.2 *M*) and 0.2 *M* acetate buffer of pH 4.6 (I=0.2). The substances gave single symmetric boundaries with nearly the same mobilities in either of the buffers. The boundary spreading of the cancerous mucopolysaccharide was pretty reversible. See Fig. 1.

Ultraviolet absorption. 50 mg% solutions (pH 6.4) were examined in a photoelectric spectrophotometer (EPB-U type, Hitachi). Cell 1 cm. thick. The mucopolysaccharides gave both a spectrum with a low peak at 270 mμ, suggesting a trace of cyclic amino acids, but there occurred no particular absorption at 260 mμ characteristic of nucleic acids. See Fig. 2.

* 217th report of Masamune and co-workers' " Biochemical Studies on Carbohydrates." Read before the XVIth General Meeting of the Japanese Cancer Association, Osaka, October 30-31, 1957.

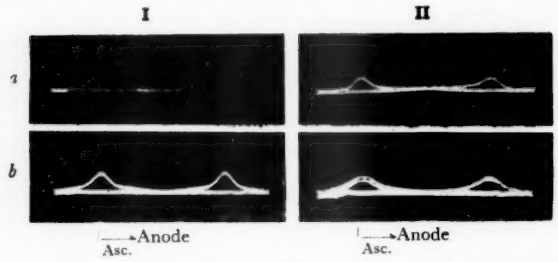

Fig. 1. Electrophoretic patterns (2/3 ×) of 1% solutions of the cancerous (*a*) and non-cancerous (*b*) MPSs II in 0.1 *M* veronal-HCl buffer of pH 8.6 and I 0.1+NaCl (0.2 *M*) (**I**) and 0.2 *M* acetate buffer of pH 4.6 (I 0.2) (**II**). Current 8 mA; temperature 15°C; exposed 120 min. after starting current. Mobilities in $cm^2 \cdot volt^{-1} \cdot sec^{-1} \times 10^{-5}$:

Cancerous MPS II in veronal-HCl buffer $\dfrac{(-0.30) \times 0.01278 \times 0.3}{0.008 \times 7200} = -2.0$

in acetate buffer $\dfrac{(-0.10) \times 0.00588 \times 0.3}{0.008 \times 7200} = -0.3$

Non-cancerous MPS II in veronal-HCl buffer $\dfrac{(-0.28) \times 0.01278 \times 0.3}{0.008 \times 7200} = -1.9$

in acetate buffer $\dfrac{(-0.13) \times 0.00588 \times 0.3}{0.008 \times 7200} = -0.4$

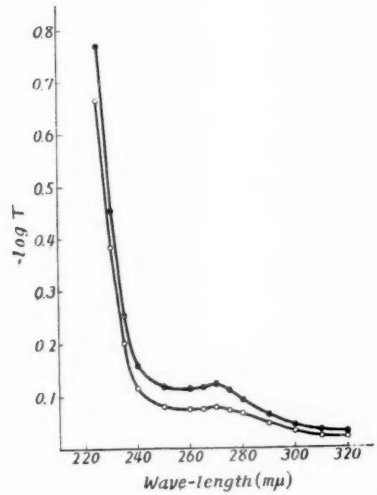

Fig. 2. Ultraviolet absorption spectra of aqueous 50 mg% solutions of the cancerous (-○-○-) and non-cancerous (-●-●-) MPSs II.

Solubility. The two substances behaved similar also in this respect. They dissolve in water of any pH value, 90% (v/v) phenol, saturated $(NH_4)_2SO_4$ (room temperature) and saturated Na_2SO_4 (33°C), and are not precipitated from water with lead acetate, cupric chloride, barium acetate, baryta and trichloroacetic acid. They are insoluble in glacial acetic acid, ethanol, acetone and ether.

Test-tube tests. No dissimilarity was found between the substances in the following test-tube tests. Molisch (卌), Bial (+), biuret (+), Osaki-Turumi (direct as well as indirect) (+), Neuberg-Saneyoshi (−), Dische (−), tests for phosphorus and hydrolysable sulfur (−).

Paper partition chromatography of components. 1) Sugars. 8 mg. substance was hydrolyzed with 1 *N* H_2SO_4, and the hydrolysate chromatographed in a manner similar to the one in the Second Report.[2] Glactosamine, glucosamine, galactose, glucose, mannose and L-fucose were detected but no pentose in both of the substances from the cancerous and non-cancerous materials. See Fig. 3.

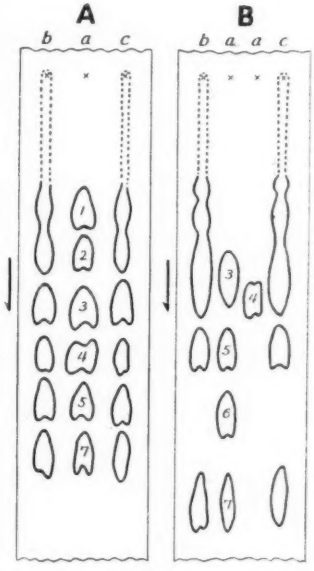

Fig. 3. Sugar paper chromatograms of MPSs II developed by descending irrigation with solvent butanol-pyridine-water (5:3:2 by volume) (**A**) or with solvent *n*-butylacetate-acetic acid-ethanol-water (3:2:1:1 by volume) (**B**) at 25°±1°C for 18 hrs. Paper slivers: Toyo Roshi filter paper No. 2, 10×60 cm. Spray reagent: 2% aniline phthalate in moist butanol. Reference runs were carried out using 0.02 cc. of 1% solutions with respect to individual sugars.

a: reference run. *b*: the hydrolysate of the cancerous MPS II. *c*: the hydrolysate of the non-cancerous MPS II. *1* galactosamine, *2* glucosamine, *3* galactose, *4* glucose, *5* mannose, *6* xylose, *7* L-fucose.

2) Amino acids. 6 mg. substance was subjected to 6 *N* HCl-hydrolysis and following operations described in the Second Report.[2] Fig. 4 is a reproduction of the chromatogram of the cancerous MPS II after hydrolysis. The hydrolysate of the non-cancerous MPS II gave a similar chromatogram. Namely, either of the two MPSs II proved to contain

aspartic and glutamic acids, serine, glycine, hexosamine, lysine, histidine, arginine, threonine, alanine, tyrosine (weak spot), proline, valine and/or methionine and leucine and/or isoleucine and/or phenylalanine.

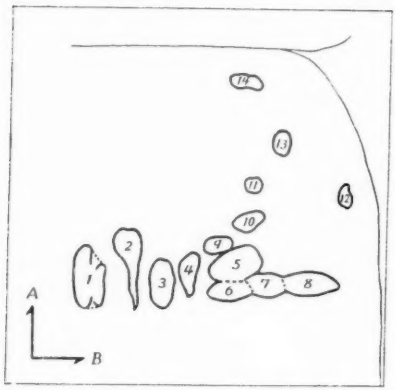

Fig. 4. Amino acid paper chromatogram of the cancerous MPS II. Paper square (Toyo Roshi filter paper No. 50) 30×30 cm. (A) Primary irrigation with solvent butanol-acetic acid-water (4:1:2 by volume) (13 hrs., 25°±1°C). (B) Secondary irrigation with solvent 80% phenol in ammonia-containing atmosphere (13 hrs., 25°±1°C). Spray ragent 0.2% ninhydrin in moist butanol.

1 aspartic acid, *2* glutamic acid, *3* serine, *4* glycine, *5* hexosamine, *6* lysine, *7* histidine, *8* arginine, *9* threonine, *10* alanine, *11* tyrosine, *12* proline, *13* valine and/or methionine, *14* leucine and/or isoleucine and/or phenylalanine.

Quantitative analysis. The analytical figures obtained are embodied in Table I. Nitrogen-, total-hexosamine-, galactose- and mannose-contents and iodine uses don't noticeably differ between the cancerous and non-cancerous MPSs II. On the contrary, glucosamine/galactosamine ratios and glucose-, sialic acid- and L-fucose-contents are discrepant from substance to substance, the cancerous MPS surpassing in the first three points and falling behind the non-cancerous in the last one point.

Optical rotation. The watery solutions examined were of pH 6.8. The two substances were found levorotatory to resembling extents.

$$\text{Cancerous MPS II} \quad [\alpha]_D^{18} = \frac{-0.195 \times 1}{0.0100 \times 1} = -19.5°$$

$$\text{Non-cancerous MPS II} \quad [\alpha]_D^{18} = \frac{-0.228 \times 1}{0.0120 \times 1} = -19.0°$$

Blood group potency. The glass plate method of Masamune *et al.*[8] was applied. The results are shown in Table II. The cancerous substance

TABLE I

Composition of MPSs II of Stomach Cancer and of Stomach Mucosa

Analysis	In per cent		In equivalents per equivalent weight	
	Stomach cancer	Stomach mucosa	Stomach cancer	Stomach mucosa
N*	5.3	5.6	3.63	3.68
Total hexosamine†	27.0	28.9	1.44	1.48
$\frac{\text{Glucosamine}}{\text{Galactosamine}}$ ratio†	4.4	3.3		
Galactose‡	18.8	19.6	1.0	1.0
Glucose‡	7.3	2.9	0.39	0.15
Mannose‡	6.3	6.7	0.34	0.34
L-Fucose§	6.9	10.8	0.40	0.61
Sialic acid//	11.0	6.7	0.34	0.20
Ash¶	2.1	1.6		
Iodine use as glucose**	5.2	5.2		

* Micro Kjeldahl. † Masamune and Yosizawa.[3] ‡ Masamune and Sakamoto.[4] § A modification of Dische and Shettles.[5] // Direct Ehrlich reaction of Werner and Odin ;[6] Calcd. as $C_{11}H_{19}NO_9$. ¶ Pregl method without use of conc. H_2SO_4. ** Macleod and Robison.[7]

TABLE II

Anti-hemagglutinative Potency of MPSs II

Goat: normal anti-O goat serum. Anti-E: eel serum. Anti-OS: anti-OS saliva chicken serum. − no hemagglutination, ± faint hemaggl., + slight hemaggl., ++ marked hemaggl., ## strong hemaggl.

MPS II	Red cells	Serum	Dilution of the substances ($1:10^3\times$)										
			1	2	4	8	16	32	64	128	256	512	∞
Cancerous	A	α	##	##	##	##	##	##	##	##	##	##	##
	B	β	##	##	##	##	##	##	##	##	##	##	##
	O	Goat	+	##	##	##	##	##	##	##	##	##	##
	O	Anti-E	##	##	##	##	##	##	##	##	##	##	##
	O	Anti-OS	##	##	##	##	##	##	##	##	##	##	##
Non-canc.	A	α	−	+	++	##	##	##	##	##	##	##	##
	B	β	+	++	##	##	##	##	##	##	##	##	##
	O	Goat	−	++	##	##	##	##	##	##	##	##	##
	O	Anti-E	±	++	##	##	##	##	##	##	##	##	##
	O	Anti-OS	−	−	++	##	##	##	##	##	##	##	##

TABLE III

Anemia-inducing Potency of MPSs II

No. of assay	Rabbit: No.	Rabbit: Body wt. (kg.)	Rabbit: Sex	Material intravenously injected	Dose per kg. body wt. (mg.)	Number of red cells ($10^4\times$; per cmm.): Before injection	Time after injection (hrs.): 1	2	3	4	5	Maximum decrease (%)
1	7	2.2	♂	Cancerous MPS II	1	746	745	725	723	751	**712**	5
2	8	2.7	♀		„	585	607	592	608	613	596	
3	9	2.7	♂		3	662	668	662	658	**633**	670	4
4	10	2.7	♂		„	679	694	651	**645**	678	653	5
5	11	2.3	♂		„	652	664	674	647	**621**	625	5
6	12	2.5	♂	Non-cancerous MPS II	3	639	654	643	638	632	**622**	3
7	13	2.7	♀		„	602	595	**570**	571	576	580	5
8	14	2.3	♀		„	600	**578**	600	583	593	605	4

TABLE IV

Liver-catalase Depression *in vivo* of MPSs II

In each test-set, the average volume of O_2 evolved for each dose is expressed in per cent of the corresponding volume of O_2 in the blank control.

No. of test-set	No. of mice used	Amount of the substances intraperitoneally injected (mg.): Cancerous MPS II 5	3	1	Non-cancerous MPS II 5	3	1
1	9			91			96
2	15	90		97	98		97
3	15	96	96		103	100	
4	15	90	97		92	105	

exhibited no group potency. That the non-cancerous was slightly inhibitory to the agglutination of A and O erythrocytes is most probably due to faint contamination with the very strong group mucopolysaccharide or mucopolysaccharides.

Anemia-inducing potency (*KIK activity*). It was examined as by Masamune and Kawasaki.[9] As shown in Table III, the substances caused no more decrease than 5% of number of erythrocytes, even when injected into a rabbit in dosage of 3 mg. per kg. body weight.

Toxohormone activity. It was determined according to Masamune and others[10] (gasometry of catalase activity). Injection of even 5 mg. amounts of MPSs II did not entail depression of liver catalase in mice. See Table IV.

SUMMARY

1. MPSs II, cancerous and non-cancerous, in the First Report were examined physically, chemically and biologically.

2. They proved homogeneous electrophoretically and absorbed no ultraviolet light near 260 mμ.

3. They are made up of same components.

4. However, glucosamine/galactosamine ratio and contents of glucose, of sialic acid and of L-fucose vary from substance to substance.

5. Their optical rotations resemble each other.

6. They don't possess group activity within ABO classification. They are also devoid of KIK- and toxohormone-activity.

Through the Grant Committee for Scientific Researches the Ministry of Education gave a grant in aid to us, which is gratefully acknolwedged. H. Masamune.

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The Tohoku Journal of Experimental Medicine, Vol. 68, No. 2, 1958

Biochemical Studies on Carbohydrates

CCXVIII. N-Acetyl-4,6-benzylidene-glucosamine

By

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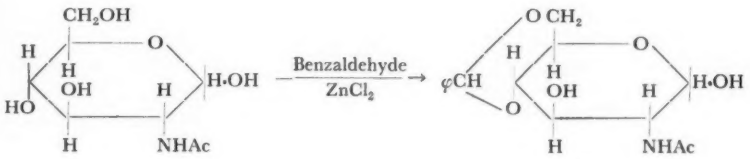
(Received for publication, April 3, 1958)

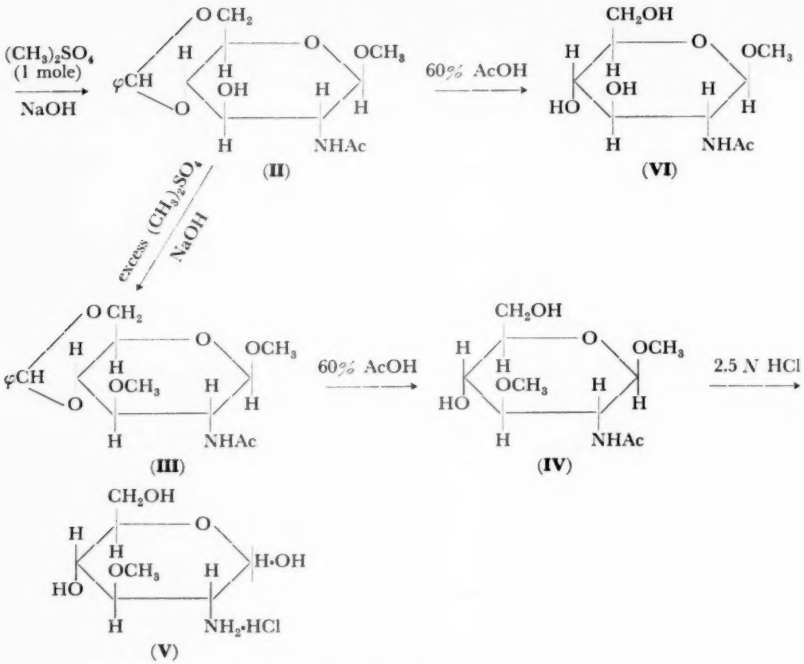
N-Acetylglucosamine was condensed with benzaldehyde under the conditions, which Wood[1] had preferred for synthesis of 4,6-benzylidene-glucose, with a yield reaching 90% of theory. The product (**I**) reduced Fehling reagent and consumed the calculated amount of iodine. Hence, in it, the glycosidic OH of N-acetylglucosamine is not participant in the condensation. It further proved to possess another OH in a free state. By successive methylations with dimethylsulfate, a monomethyl (**II**) and a dimethyl (**III**) derivative were produced. And hydrolysis of the latter derivative with 60% acetic acid gave a methylglycoside of N-acetyl-monomethyl-glucosamine (**IV**), which changed into a monomethylglucosamine hydrochloride (**V**) in turn on boiling with 2.5 *N* HCl. **V** melted and rotated D-light identically as 3-methyl-glucosamine hydrochloride (Neuberger[2]) did, and mixed melting point test showed no depression. **IV** reduced neither alkaline iodine nor periodate, but was levorotatory contrary to the dextrorotatory N-acetyl-3-methyl-α-methyl-glucosaminide (Neuberger[2]), so that it is assumed as N-acetyl-3-methyl-β-methyl-glucosamino-pyranoside.

Therefore **I** is concluded to be N-acetyl-4,6-benzylidene-glucosamine.

II gave N-acetyl-β-methyl-glucosaminopyranoside (Zilliken *et al.*[3]) (**VI**) when hydrolyzed with hot 60% acetic acid.



(**I**)



Experimental

N-Acetyl-4,6-benzylidene-glucosamine. 10 g. of fine powdered anhydrous N-acetylglucosamine, 40 g. of freshly fused and powdered zinc chloride and 200 cc. of freshly distilled benzaldehyde were shaken together for 12 hours at room temperature. The mixture was poured into 250 cc. of iced water and stood for several hours. The white pasty precipitate was kneaded with 200 cc. of iced water, filtered off and agitated in 500 cc. of petroleum ether, and the insoluble crystalline residue was brought onto a suction funnel.

It was heated with 1.5 *l.* of abs. methanol under reflux on boiling water until it dissolved entirely, and filtered quickly while hot. The filtrate was distilled in vacuo to about 300 cc. and placed in a refrigerator. The crystalline deposit was washed with ice-chilled methanol. About 8 g. of crystals (soft long needles) was yielded. Recrystallization from abs. methanol did not elevate the melting point. From the mother fluids was obtained about 4 g. of crystals of similar appearance. F.P., 214–215° (decomp.); $[\alpha]_D^{15}$ +10.0° (c 1.0; pyridine; final). Found: N, 4.55; CH_3CO, 14.02; OH, 10.72; iodine use as glucose, 57.0. $C_{15}H_{19}O_6N$ requires N, 4.53; CH_3CO, 13.90; OH, 11.00; iodine use as glucose, 58.00. Soluble in pyridine, dioxane, alkaline methanol and ethanol and hot plain methanol. Slightly soluble in hot ethanol and $CHCl_3$. Insoluble in water.

N-Acetyl-4,6-benzylidene-β-methyl-glucosaminide. 5 g. of N-acetyl-4,6-benzylidene-glucosamine was dissolved in 50 cc. of ice-chilled methanolic 2 *N* NaOH, and after addition of 1.8 cc. of dimethylsulfate, shaken under continuous cooling until it reduced Fehling reagent no more, followed by pouring into 500 cc. of iced water. After several hours the mixture containing some deposit was filtered. The filtrate was concentrated, and when crystals began to appear, it was placed in a refrigerator to complete crystallization. The deposit (crystals in long needles) was recrystallized from 50 cc. of methanol. Yield 2 g. (38% of theory). F.P. 252–253° (decomp.). $[\alpha]_D^{18}$ −40° (c 1.0; $CHCl_3$). It did not reduce Fehling reagent and smelled of benzaldehyde on boiling with 60% acetic acid or 1 *N* HCl. Found: N, 4.26; CH_3CO, 12.9; OH, 5.42; CH_3O, 10.25. $C_{16}H_{21}O_6N$ requires N, 4.33; CH_3CO, 13.6; OH, 5.27; CH_3O, 9.6.

N-Acetyl-4,6-benzylidene-3-methyl-β-methyl-glucosaminide. 3.5 g. of N-acetyl-4,6-benzylidene-β-methyl-glucosaminide was dissolved in 150 cc. of dioxane, warmed to 50°C and treated with ten portions of 15 cc. dimethylsulfate and of 33 cc. 30% NaOH at 10-minute intervals. After standing for a few hours at room temperature, the mixture was poured into a large volume of iced water, and the crystalline precipitate was filtered off with suction. The crystalline material was recrystallized from ethanol. Soft fine needles weighing 2.9 g. (79% of theory) were yielded. F.P. 208–209° (decomp.). $[\alpha]_D^{15}$ −76.0° (c 1.0; $CHCl_3$). Found: N, 4.1; CH_3O, 17.6. $C_{17}H_{23}O_6N$ requires N, 4.2; CH_3O, 18.4.

N-Acetyl-3-methyl-β-methyl-glucosaminide. 0.65 g. of N-acetyl-4,6-benzylidene-3-methyl-β-methyl-glucosaminide was suspended in 15 cc. of 60% acetic acid and refluxed on boiling water. The clear solution given in 7 minutes was further heated for 15 minutes. When cold it was diluted with three volumes of water, and after exhaustion with three 15 cc. portions of chloroform, distilled in vacuo, and the crystalline residue was recrystallized from 5 cc. of ethanol. 0.30 g. (62.5% of theory) of crystals in fine needles was given. F.P. 164–165°. $[\alpha]_D^{17}$ −58.4° (c 1.4; H_2O). Found: N, 5.8; CH_3O, 24.5; OH, 11.2. $C_{10}H_{19}O_6N$ requires N, 5.6; CH_3O, 24.9; OH, 10.1. The substance did not reduce alkaline iodine and sodium metaperiodate.

3-Methyl-glucosamine hydrochloride. 2 g. of N-acetyl-3-methyl-β-methyl-glucosaminide was hydrolyzed by heating with 50 cc. of 2.5 *N* HCl under reflux at 100°C for 4 hours. The brown solution was decolorized with charcoal, and the filtrate was concentrated to dryness, dissolved in 15 cc. of hot methanol and allowed to cool. Crystallization was processed by addition of acetone in small portions—fine needles. Yield 1.2 g. (60% of theory). F.P. 214–215° (decomp.). $[\alpha]_D^{23}$ +120° (5 min.), +92.0° (10 h.; eq. val.) [c 1.2; H_2O]. Found: N, 6.1; CH_3O, 12.7. $C_{17}H_{15}O_5N{\cdot}HCl$ requires N, 6.1; CH_3O, 13.5. This product melted also at 214–215°, when mixed with the one prepared according to Neuberger.[2]

N-Acetyl-β-methyl-glucosaminide. 1 g. of N-acetyl-4,6-benzylidene-β-methyl-glucosaminide was hydrolyzed with 60% acetic acid and further treated as N-acetyl-4,6-benzylidene-3-methyl-β-methyl-glucosaminide was. The crystals given—needles—weighed 0.47 g. (64.5% of theory). F.P. 197–198°. $[\alpha]_D^{20}$

$-42.5°$ (c 1.0; H_2O).

My thanks are due to the Ministry of Education for a grant given through the Grant Committee for Scientific Researches. H. Masamune.

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The Tohoku Journal of Experimental Medicine, Vol. 68, No. 2, 1958

Effects of Hyperpnea on Values of Electric Flicker

By

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(Received for publication, April 5, 1958)

Introduction

It has been previously reported by Motokawa, Iwama and Suzuki[1),2)] that Motokawa's method of electric flicker can be used as a very convenient indicator for the measurement of fatigue and that it was very sensitive to oxygen lack. Suzuki and co-workers[4),5)] have further investigated the effect of a small dose of roentgen ray upon the human body, the effects of injection of analgetics, and those of blood transfusion and bleeding on values of electric flicker (EFV). Moreover, in our previous paper[6)] the effect of low oxygen inhalation on EFV was reported in comparison with arterial oxygen saturation. In this investigation we had a chance to observe the effect of hyperpnea on the electric flicker values. Gellhorn and Spiesman[7)] investigated the influence of hyperpnea and oxygen lack upon hearing, and reported that, immediately after a period of hyperpnea, hearing was greatly decreased as a result of acapnia.

In the present experiment the effect of hyperpnea upon EFV was investigated more systematically. In addition, the value of arterial oxygen saturation was measured by means of an ear oximeter.

Experimental

Method

The subjects in the present experiment were 5 healthy males. For 30 minutes before each experiment, the subjects were laid down quietly on the bed, breathing the room air. The measurements of EFV and arterial oxygen saturation were carried out for 10 minutes after the rest to obtain control values. The subjects were subjected to acapnia caused

by hyperpnea after the measurement of the control values. Similar measurements were made continuously during and after deep breathing. The subjects inhaled as deeply as possible for 5 minutes according to the rhythm of a metronome. In this experiment the frequency of the metronome beat was about 25 per minute.

The apparatus for electric flicker used in the present experiment was the same as was described in Motokawa's report.[3] The stimulating current was increased and decreased automatically, to determine the threshold for appearance of flicker and that for its disappearance. The difference of both thresholds, S_1–S_2, was denoted by EFV. The EFV was expressed in terms of μA.

The apparatus used for the measurements of arterial oxygen saturation was the ear oximeter of Wood type.[8]

Results

The results obtained from 3 subjects, who performed deep breathing for 5 minutes are represented in Fig. 1 (I, II and III). As can be seen in

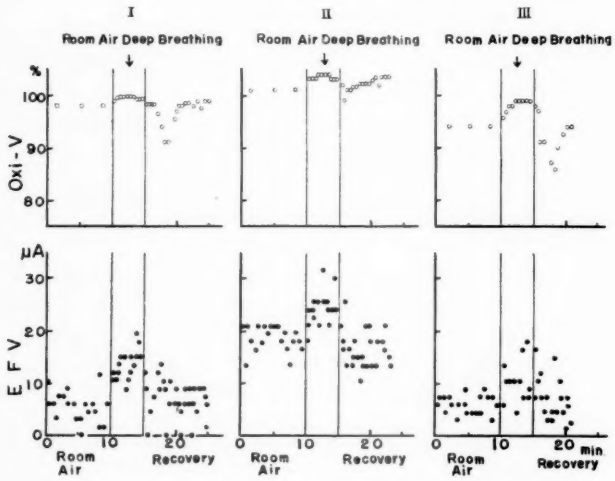

Fig. 1. Changes of electric flicker value (EFV) and ear oximeter value (Oxi-V) by deep breathing of room air. I, II and III refer to different subjects.

this figure, the results were consistent throughout the whole series of experiments and showed that after the beginning of hyperpnea EFVs were remarkably increased, and oximeter values (Oxi-V) also increased. Recovery was very prompt with regard to the EFV, but as to the Oxi-V it was characteristic that a recovery to the initial value, was followed by a

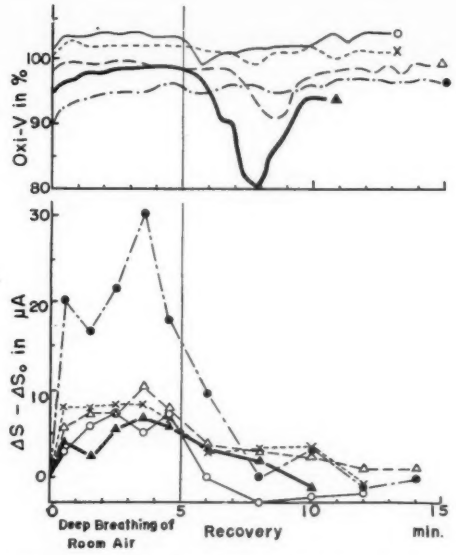

Fig. 2. Changes of EFV and Oxi-V obtained from 5 subjects during and after deep breathing of air. ΔS_0: Initial EFV. ΔS: EFV during and after deep breathing.

temporary decrease of Oxi-V below the initial value. It is obvious that the difference between the EFVs before and during hyperpnea was statistically significant. Fig. 2 shows the results obtained from similar measurements on other 5 subjects.

In the following experiment, deep breathing of pure oxygen was performed for 3 minutes. The result obtained is plotted in Fig. 3. As can be seen in this figure, EFV increased during hyperpnea of 100% O_2, and Oxi-V also increased remarkably. After cessation of deep breathing of pure oxygen the recovery occurred gradually with Oxi-V as well as with EFV. This is a remarkable contrast with the prompt recovery of EFV after deep breathing of the ordinary air.

In the following experiment, the measurements of EFV and Oxi-V were made before, during and after normal breathing of pure oxygen. The results obtained are shown in Fig. 4. As can be seen in this figure, Oxi-V increased during normal breathing of 100% O_2, but no significant difference could be found between the EFVs obtained before and during the normal breathing of pure oxygen. From this fact, it may be said that the increase of EFV during hyperpnea is not due to a surplus inhalation of oxygen.

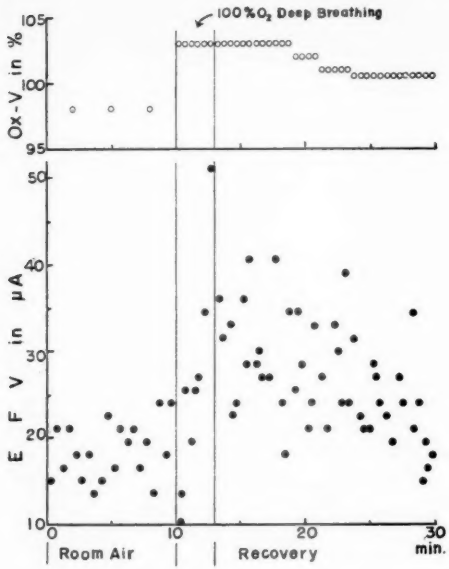

Fig. 3. Changes of EFV and Oxi-V by deep breathing of pure oxygen.

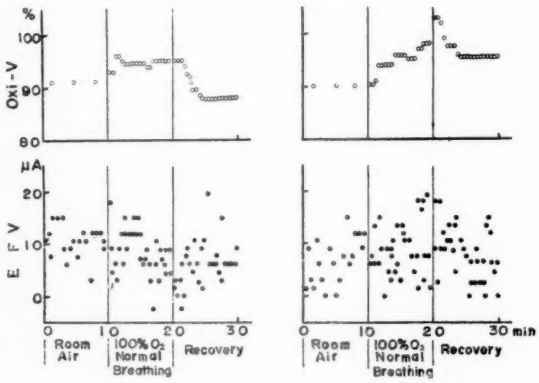

Fig. 4. Effects of normal breathing of 100% O_2 upon EFV and Oxi-V.

Next, the effect of deep breathing of a mixture of 1% CO_2 and 99% O_2 was investigated. The result obtained is shown in Fig. 5. As can be seen in this figure, some slight increase occurred during deep breathing of this gas mixture with regard to Oxi-V, but no appreciable change could be found with regard to EFV. In other 3 subjects, similar measurement

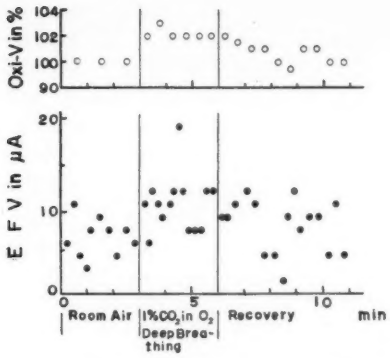

Fig. 5. Effect of deep breathing of 1% CO_2 in O_2 upon EFV and Oxi-V.

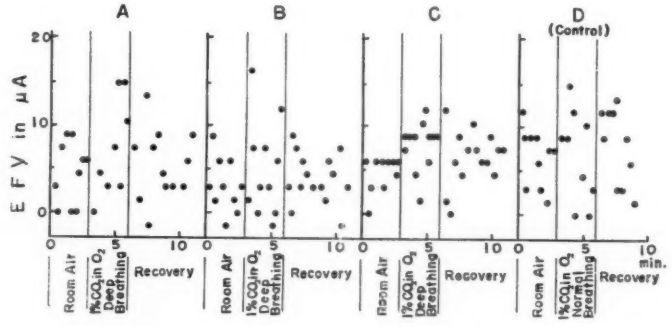

Fig. 6. Experiments showing effect of 1% CO_2 for preventing increases of EFV by deep breathing. A, B and C refer to different subjects. D: control for normal breathing of 1% CO_2 in O_2.

were made. The results obtained are plotted in Fig. 6 (A, B and C). In these experiments no statistically significant change could be found either. As control, normal breathing of the same gas mixture was made in Fig. 6 D, which shows no effect on EFV. From what has been shown above it is apparent that with administration of 1% CO_2 we can avoid the effect of hyperpnea upon EFV.

Discussion

In the previous experiment[6] Oxi-V decreased and EFV increased by inhalation of 10% oxygen. But in the present experiment both EFV and Oxi-V increased by hyperpnea as can be seen in the Figs. 1, 2 and 3. From these observations the following question arises: To which factor,

surplus oxygen inhalation or acapnia, the increases of both EFV and Oxi-V by hyperpnea are to be ascribed? It is not difficult to ascribe the increase of Oxi-V during hyperpnea to surplus oxygen inhalation, but the increase of EFV during hyperpnea may not be due to surplus oxygen inhalation, because the EFV shows no increase during normal breathing of 100% O_2. As can be seen in Fig. 4, the Oxi-V increased during normal breathing of pure oxygen, but with regard to EFV no change occurred. The fact that deep breathing of 1% CO_2 in O_2 caused no increase in EFV suggests the importance of acapnia. Our result provides evidence that the EFV is sensitive to acapnia. A higher concentration of CO_2 may have some effect upon the EFV, although no experiment was carried out with higher concentration of CO_2 in the present study.

Simonson[9] reported that breathing 5% CO_2 in O_2 was accompanied by a fall of 4 cps in FFF, and CO_2 in concentration below 4% has no appreciable effect on FFF. According to Gellhorn and his coworker,[7] the threshold concentration of CO_2 to maintain auditory, visual and nystagmic responses in normal state was about 4%. Gellhorn and Spiesman[7] investigated the influence of hyperpnea upon hearing, and reported that a decrease in hearing may last as long as 10 minutes after a period of hyperpnea. They discussed that the change in hearing during low oxygen inhalation and after hyperpnea might be due to some chemical cellular alterations in the central nervous system.

At present, it is difficult to explain the mechanism in which the EFV is increased by hyperpnea, but it can be said that the method of electric flicker represents one of the most sensitive methods to acapnia.

Summary

By means of Motokawa's method of electric flicker (EF), we measured variations of electric flicker values (EFV) of 5 subjects caused by hyperpnea, the inhaled gases being air, 100% O_2 and 1% CO_2 in O_2. Measurements by an ear oximeter were carried out in parallel.

1. During 5 minutes' deep breathing of air, both EFV and oximeter values (Oxi-V) increased.

2. Also by deep breathing of pure oxygen, both kinds of values increased.

3. In case of 100% O_2 inhalation (normal breathing), Oxi-V increased, but no change occurred in EFV.

4. In case of hyperpnea of 1% CO_2 in O_2, Oxi-V increased, while no change could be found in EFV.

From these findings it was concluded that the change in EFV by hyperpnea is due to acapnia.

The authors have pleasure in acknowledging their indebtedness to Prof. K. Motokawa who has guided them kindly.

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The Tohoku Journal of Experimental Medicine, Vol. 68, No. 2, 1958

Electrophoretic Studies of Liver Protein

Report I

Studies on the Experimental Methods

By

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(Received for publication, April 10, 1958)

Sato, one of the present authors, who has been engaged in electrophoretic studies of the liver protein, has devised a method of plotting its electrophoretic patterns, using liver extracts obtained from small specimens of tissue sampled from the liver of patients of diseases in the upper abdomen at laparotomy and has published reports on the method and the results clinically obtained thereby in a previous issue of this J. Exp. Med.[1),2)]

More recently, the authors detected a biochemical defect in his experimental method previously reported and has perfected another method simple in clinical application and has made several reports on it.[3)-8)]

In the present paper, the authors will report 1) on the experimental method itself (treatment of the liver specimen and the electrophoretic process of its extract), 2) on the experimental results concerning the biochemical characteristics of the fractions appearing in the electrophoretic patterns and 3) on some results of our review on the value of the experimental method, in the following.

Experimental

Methods

The specimen of about 3 g. cut out from the margin of the liver is immediately shredded, wrapped up in clean cotton gauze to wring out its blood content thoroughly, resliced into thinner sections and dried under vacuum in an ice-box kept below 5°C. For facilitating desiccation, the fine liver slices are first smeared on filter paper and put into the desiccator. Usually, the desiccating process is completed in 24 hours or so. Then, the slices are ground in a mortar into powder, which it fit for preservation.

Then 0.5 g. of this dry powder is stirred in an extracting solvent of 5 cc.

of phosphate buffer (pH 7.8) and about 3 cc. of a yellow transparent solution of the soluble components is prepared. After dialyzing this solution with pH 7.8 phosphate buffer, it is subjected to electrophoresis for about 60 min. (Table I), under the conditions of 100 Volt—10 mA ; pH 7.8 ; 1/20 M phosphate buffer ; ionic strength, 0.14 ; 15°C. The final protein concentration of the extract stands at about 2.0 g/dl (by Kjeldahl's method).

In Fig. 1 are shown several electrophoretic patterns obtained by this method.

TABLE I

Treatment of the Liver Specimen

Fresh liver (2–3 g.)
↓
Shredding
↓
Dried in vacuo (in a desiccator, 0–5°C)
↓ Finely ground
Dry powder (ca. 500 mg)
↓ pH 7.8 buffer, 5 cc
Extract
↓
Dialysis (24 hrs. against pH 7.8 buffer)
↓
Electrophoresis (100 V., 10 mA., 15°C)

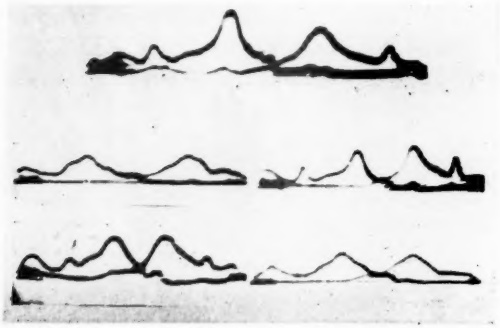

Fig. 1. Electrophoretic patterns of liver extracts.

Biochemical Characterization of Each Fraction

In interpreting the peaks (determining their biochemical characteristics), we proceeded by making various tests in comparison with the patterns of human plasma, always using paper electrophoresis.

First, we studied in collation the electrophoretic patterns on filter papers

obtained with a specimen of liver extract as shown in Fig. 2 and with human plasma. We see in Fig. 2 that in the liver extract is found scarcely any trace of the fraction with the mobility equal to that of albumin in plasma, and that the largest part of the protein fractions in the liver extract consists of those with

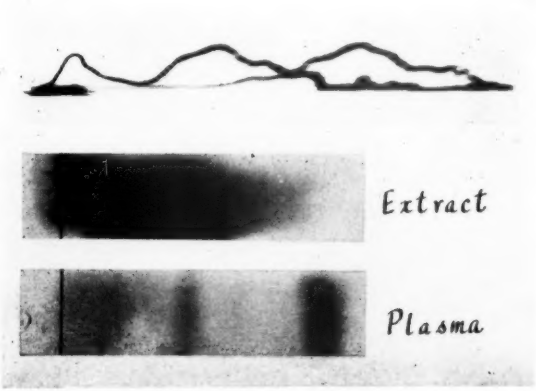

Fig. 2. Electrophoretic pattern and filter-paper electropherogram of liver extract.

the mobility of α-, β- and γ-globulin. In this case, the protein concentration of the liver extract was ca. 2 g/dl by Kjeldahl's method, the buffer was 7.8 in pH value, the ionic strength 0.14, the concentration of the phosphate buffer 1/20 M, the current density 0.3 mA./cm., the temperature 15°C and the electrophoresis was continued for about 16 hours. Toyo filter paper No. 51 was used and for detecting protein, staining by B.P.B. method was adopted.

Next, we mixed the liver extract with human plasma and studied the filter-paper electropherogram and the electrophoretic pattern of such mixtures.

First we took sample of human plasma and liver extracts both of 3 g/dl protein concentration (as estimated by Kjeldahl's method), then mixed the two liquids in equal parts, and studied the filter-paper electropherograms of these three liquid samples. The conditions adopted were the same as above.

As shown in Fig. 3 by the graph obtained with the plasma-liver extract mixture, we learn that the protein fraction at the position of albumin in the plasma is little affected by the mixture and that most of the protein fractions in the liver extract coincide in the positions of α-, β-, and γ-globulins, especially precisely in the position of β-globulin in the plasma, indicating nearly the same mobility as these globulin in human plasma.

This finding also show that the largest majority of the protein fractions in the liver extract have the mobility commensurate with that of plasma globulins.

The same relation between the protein fractions in the liver extract and plasma was reviewed, using electrophoretic patterns obtained with a Tiselius apparatus.

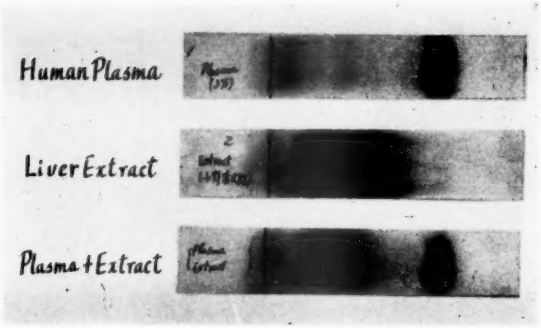

Fig. 3. Filter-paper electropherogram of the plasma-liver extract mixture.

Samples of liver extract and human plasma, all showing the protein concentration of 2 g/dl by Kjeldahl's method were electrophorized under the same conditions (phosphate buffer of pH 7.8 and ionic strength 0.14; current, 100 V, 10 mA; 15°C; 60 min.) separately and in 1:1 mixture, to examine the 3 electrophoretic patterns in comparison.

The patterns obtained are shown in Fig. 4. In the pattern of the extract-plasma mixture, we see the peak indicating albumin in the plasma markedly lowered due to dilution, but the peaks indicating α-, β-, φ- and γ-globulins, especially of β-globulin, are much higher than those in the electrophoretic pattern of pure plasma.

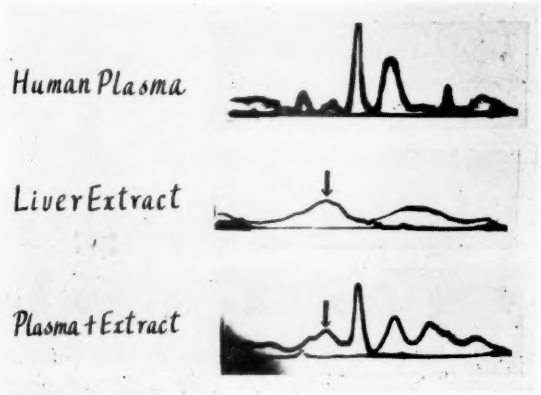

Fig. 4. Electrophoretic pattern of the plasma-liver extract mixture.

This result seems to mean that the majority of the peak produced in the electrophoretic pattern of the liver extract overlap with those produced by α-, β-, φ- and γ-globulins in plasma, indicating that these two sets of fractions have the same respective mobility.

Next, we have studied this subject by means of the measured values of the different fractions. As shown in Table II, the albumin value in the human plasma was 59.2%, while that in the 1:1 mixture of plasma and the liver extract was markedly reduced to 36.5%, but α-, β-, and φ-globulin values were found increased to 10.3, 29.2, and 10.3% respectively in the liver extract, β-globulin in particular showing a marked rise in content. The value of γ-globulin alone was found decreased a little.

TABLE II

Fraction Values of Plasma-Liver Extract Mixture

	Alb. (%)	α (%)	β (%)	φ (%)	γ (%)
Plasma	59.2	7.8	9.4	7.8	15.8
Plasma + Extract (1:1 mixture)	36.5	10.3	29.2	10.3	13.5

From the above, we can conclude that the electrophoretic patterns obtained by our method reveal that the liver extract contains little or no fraction corresponding to albumin found in blood plasma but mainly consists of globulin fractions corresponding in their postitions to α- and β-globulins in plasma.

For further assuring the above fact, the authors next examined the similarity of the electrophoretic pattern and the curve plotted with the values obtained by measurements on the filter-paper electropherogram of the same liver extract.

As shown in Fig. 5, these two curves were found to show a striking similarity in form.

Upon closer examination of the curve obtained by filter-paper electrophoresis, we found that it also showed the existence of the 5 fractions cited above, as may be seen by comparison of the curve for plasma obtained under the same condition, as indicated in a broken line in the figure.

Thus, in the electrophoretic pattern of human liver extract obtained by our method too, we succeeded in separating 5 protein components corresponding to the 5 fractions of albumin, α-, β-, φ- and γ-globulin arranged in this order in the electrophoretic pattern of human plasma.

Then, we tried a comparison of the measured values of the fractions albumin, α-, β-, φ- and γ-globulins, in the descending order of their electrophoretic mobility, on our electrophoretic pattern of a liver extract sample and the fraction values obtained from the filter-paper electropherogram of the same sample of liver extract.

As shown in Table III, the values were found to agree with the maximum deviation within the range of 4.5%.

Thus, it was clarified that the liver extract, as indicated by its electrophoretic pattern, consists of 5 protein fractions and that these 5 correspond to albumin, α-, β-, φ- and γ-globulins of plasma, as viewed electrophoretically. Accordingly, we will tentatively call the fractions of liver extract albumin, α-, β-, φ- and γ-globulin outright, as shown in the pattern in Fig. 6.

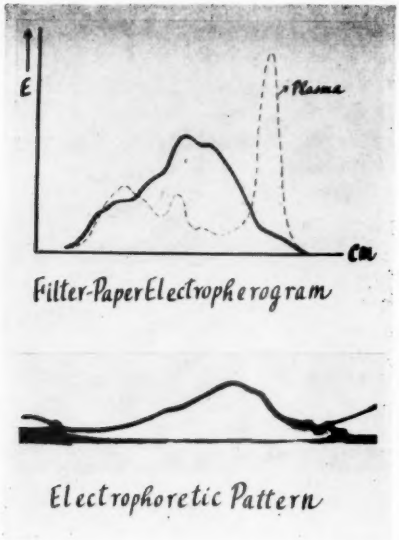

Fig. 5. Filter-paper electropherogram and electrophoretic pattern of liver extract.

TABLE III

Values of Each Fractions by Filter-Paper Electrophoresis and Tiselius Electrophoresis Compared

	Alb. (%)	α (%)	β (%)	φ (%)	γ (%)
Tiselius method	9.5	21.3	36.9	19.9	12.3
Filter-paper method	7.8	25.8	35.8	17.4	13.4
Difference	±1.7	±4.5	±1.1	±2.5	±1.1

Rescruting of the Experimental Method

On blood in the extract: As stated above, the liver specimens sampled at laparotomy were shredded and the blood content was then wrung out merely by main force, so the blood content remaining in the specimen naturally comes into the question.

The filter-paper electropherograms of the liver extract and blood plasma were, as described above, found totally different, the spot at the position indicating albumin in plasma being scarcely observable in the graph of liver extract, the latter consisting nearly of globulin fraction alone.

When a part of a specimen treated as above was rinsed in running water

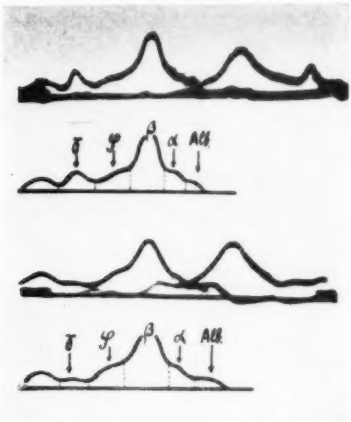

Fig. 6. Designation of the peaks as seen in the electrophoretic pattern.

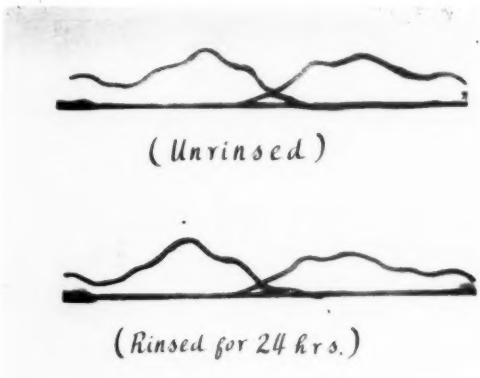

Fig. 7. Electrophoretic patterns with rinsed and unrinsed specimens compared.

for 24 hours to remove all trace of blood after shredding and wringing and its electrophoretic pattern was compared with that of the unrinsed specimen, the two patterns showed nearly the same form, as shown in Fig. 7 and the values of fractions in both the case were also very similar, as shown in Table IV, with the maximum deviation within the range of 2.1%. The total extracted protein value was a little higher in the unrinsed specimen, but the difference cannot be called significant.

From these two findings, we can deduce that in studying the electrophoretic patterns of the protein in the liver specimens obtained by our method, the blood content in them is in the order of negligible smallness.

TABLE IV

Values of Each Fractions with Rinsed and Unrinsed Specimens Compared

	Extracted protein (g/dl)	Alb. (%)	α (%)	β (%)	φ (%)	γ (%)
Unrinsed	2.3	9.3	26.5	32.4	21.6	10.2
Rinsed for 24 hours	2.0	8.6	24.4	33.5	23.5	9.1
Difference	±0.3	±0.7	±2.1	±1.1	±1.9	±1.1

On the buffer solution used: The authors have used phosphate buffer throughout our experiments, but here we will discuss the effect of veronal buffer in similar experiments.

For this purpose, we made the experiment of using veronal buffer (pH 8.6, ionic strength 0.1) as solvent for extraction and the same solution in electrophresis.

As shown in Fig. 8, the two electrophoretic patterns obtained with phosphate buffer and veronal buffer were found closely similar and no new separation of peaks was obtained with veronal. Only the electrophoretic velocity was much higher with veronal, the pattern shown in Fig. 8 being obtained within ca. 20 minutes.

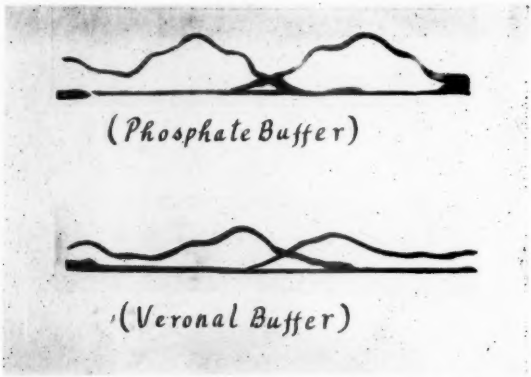

Fig. 8. Electrophoretic patterns obtained with different buffers compared.

The measured values of the fractions appearing in the patterns, as listed in Table V, were not different to a significant extent. The extracted total protein concentration also was similar in both the cases.

On the reproducibility of the electrophoretic pattern: With the specimens of the dried liver powder prepared by our method, we made two rounds of electrophoretic tests under the same conditions but on different dates. The patterns obtained thereby, as shown in Fig. 9, were found nearly similar and the measured

TABLE V

Values of Each Fractions obtained with Different Buffers Compared

	Extracted protein (g/dl)	Alb. (%)	α (%)	β (%)	φ (%)	γ (%)
Phosphate	2.3	11.7	22.4	34.6	18.6	12.7
Veronal	2.5	10.7	23.1	35.1	20.4	11.0
Difference	±0.2	±1.3	±0.7	±0.5	±1.8	±1.7

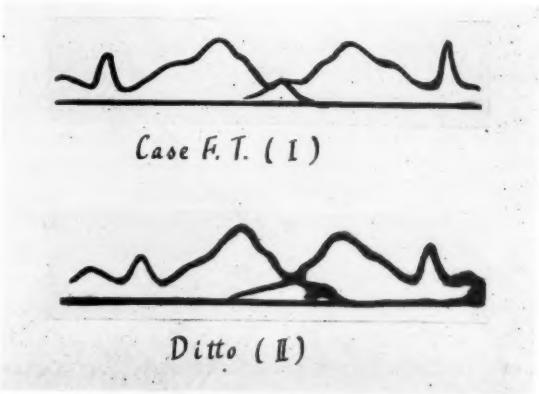

Fig. 9. Electrophoretic patterns with the same specimen compared.

TABLE VI

Differences in Measured Values with the Same Specimen

Case	Alb. (%)	α (%)	β (%)	φ (%)	γ (%)
F. Tazawa (I)	5.6	13.3	42.3	23.4	15.4
Ditto (II)	7.8	14.3	41.1	20.0	16.3
Difference	±2.2	±1.0	±1.2	±3.4	±0.9

values of the fractions were also very similar in both the cases, the maximum deviation being 3.4% (Table VI). Thus, we feel assured that the reproducibility of the results of electrophoresis under our method is utterly satisfactory.

Discussion and Conclusions

In the field of electrophoretic study of tissue protein, we have the

reports by Jacob,[9),10)] Roberts,[11)] Amberson[12)] and Blasius[13)] concerning the protein extracted from animal muscles or lymphoid tissues, but only Luck[14)] and Sorof[15),16)] have studied the liver protein by use of electrophoretic patterns. The method these authors adopted, however, were complicated and were not envisaged for clinical practice.

Since the filter-paper electrophoresis came into wider use, Demling,[17),18)] Henning[19)] and Adjutantis[20)] reported on the electrophoretic studies of the liver tissue protein or lymphoid tissue protein and Miller,[21)] Eldredge[22)] and Höhne[23)] on similar studies of the protein content of tumor tissues.

In looking through these reports, we find the electrophoretic patterns and graphs are described as variable according to the methods of tissue protein extraction and the buffer solutions used and the interpretation of the biochemical nature of the fractions detected, and consequently the names given to them has been not at all uniform by authors.

The largest obstacle, in obtaining serviceable electrophoretic patterns of tissue protein is the turbidity of the extract. Against this difficulty, there are the methods of ultracentrifugation,[15),16)] ethanol extraction under low temperature,[16),24)] mild extraction[12)] etc., but all these are complex in operation and far from being applicable in clinical practice. Formerly, as stated in previous report,[1)] Sato used to make precipitates from saline extracts, using alcohol, and thence reextracting with alkaline solutions, but since 0.5% NaOH was used as solvent, the authors had to attend to the question of possible change of protein and other tissue components thereby.

Upon reconsideration of such a difficulty, we have given up this procedure and adopted the desiccation method simple in operation in clinical practice.

The interpretation of the fractions indicated on the electropherogram has been at variance by authors, as mentioned above.

The identification of the biochemical nature of such fractions can be effected by means of several methods such as separation and qualitative test through salting out or using fractionating cells.[3)] In the present study, the authors adopted the method of studying this problem electrophoretically by collating the filter-paper electropherogram of the liver extract specimens with that of human plasma.

In an electropherogram of a sample of liver extract obtained by our method, scarcely any trace of the fraction with the electrophoretic mobility similar to albumin was found and the graph indicated that the extract mainly contained fractions with the mobility coinciding with that of α-, β-, φ- and γ-globulins alone.

This finding was confirmed also from the filter-paper electrophero-

gram of 1 :1 mixture of liver extract and human plasma of equal concentration. In such a graph, the most fractions of the liver protein components concided with those of globulins, especially, α- and β-globulins in plasma.

In the electrophoretic pattern of the same mixture, the peak at the position of albumin was reduced in height, but those standing for α-, β- and φ-globulins were found enhanced.

From the above, we concluded that the protein in liver extract consists mainly of fractions with the mobility of α-, β-, φ- and γ-globulins in plasma respectively.

In the curve obtained by plotting the measured values of the fractions appearing in the filter-paper electropherograms of liver extract, studied in collation with the same of plasma also showed that the extract contain that 5 fractions as the plasma.

Then, we assigned the peaks formed on the electrophoretic pattern of liver extract to the 5 fractions and upon comparing their relative values with those of the corresponding fractions on the filter-paper electropherogram, obtained a nearly perfect agreement.

From these results, we found that the peaks on the electrophoretic pattern of liver protein obtained by our method represented the 5 main fractions as in blood plasma, and electrophoretically examined, these 5 fractions corresponded to albumin, α-, β-, φ-, and γ-globulins making up the protein in plasma, so we presumed to name these fractions tentatively albumin, α-, β-, φ- and γ-globulins of liver protein, in the descending order of their respective mobility.

Demling *et al*[18] reported that the tissue protein of animals chiefly consists of globulins, except that in sexual organs and Luck[14),24)] and Urban[25)] reported that the liver protein mainly consists of globulins. In our present study, we have also confirmed that the liver protein contains little albumin, but is mainly made of globulins, in particular of α- and β- globulins.

In our study we have adopted the simple method of drying fresh specimens of liver tissue under low temperature in vacuo, then pulverizing them and extracting liver protein for electrophoretic analysis. Some problems may be presented concerning this procedure.

First, the problem of the blood content of the extract was studied.

Urban,[25)] Luck,[24)] Demling[19)] and Amberson[12)] tried to eliminate the blood content by perfusion of the organ or other means. In our study, we merely pressed out the blood content forcibly from the fresh shredded specimens, but found that in our filter-paper electropherogram of the extract prepared from them (Fig. 2), scarcely any trace of the fraction corresponding to albumin in blood could be detected. Again, upon rinsing

the shredded specimens in running water for 24 hours before preparing the extract, we obtain an electrophoretic pattern not at all perceptibly different from that obtained without going through the process of rinsing. The measured contents of the fractions were found not significantly changed either.

From these two findings above, we could conclude that the blood content in our specimens of liver extract was of a negligible quantity in the electrophoretic analysis we have conducted.

For extraction and electrophoresis, we used phosphate buffers of pH 7.8, but since veronal buffer is widely used as more efficient in separating the fractions, we also tried veronal buffer of nearly the same concentration (pH 8.6), but the electrophoretic pattern was closely similar to that obtained with phosphate buffer, showing little superiority for the purpose.

Adjutantis[20] says that borax buffer is better adopted for fractioning liver protein than veronal or phosphate buffer but we have not experimented with borax buffer.

We also studied the problem of the reproducibility of our results, treating parts of the same specimen on different dates by our method. The value of the fractions and the electrophoretic patterns both showed good agreement and we were assured of the satisfactory reporducibility of our electrophoretic pattern.

We cannot deny that this method of ours for plotting the electrophoretic pattern of liver protein is subject to further rescruting in many points, but we believe that the method is recommendable as convenient in clinical studies as being freen of the danger of denaturing the tissue components than the method previously reported by Sato, for this time only a buffer of low alkalinity (pH 7.8) was used, as having satisfactory reproducibility, and being comparatively easily applicable.

Summary

We have reported in the above a newly devised clinically simple and easy method of our own improving upon the method previously devised by Sato, one of the present authors, for tracing the electrophoretic pattern of liver protein.

We made investigations from several angles on the biochemical nature of the fractions appearing in the electrophoretic patterns thus obtained and arrived at the conviction that the liver protein is mainly composed of fractions electrophoretically identical with the four type of globulin found existing in human plasma. We rescrutinized our method for the interference by the blood content of the specimens, the merits of the buffer solution used and the reproducibility of the patterns, and have obtained

satisfactory conclusions on all these points.

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